GC University Lahore

The genomic basis and environment as risk factors for myocardial infarction

Name **Riffat Iqbal**

Session **2009-2012**

Roll No. **16-GCU-PHD-Z-09**

Department **Zoology**

The genomic basis and environment as risk factors for myocardial infarction

Submitted to GC University Lahore
in partial fulfillment of the requirements
for the award of Degree of

Doctor of Philosophy
in
Zoology
By

Name	**Riffat Iqbal**
Session	**2009-2012**
Roll No.	**16-GCU-PHD-Z-09**
Department	**Zoology**

DECLARATION

I, Miss. Riffat Iqbal Roll No 16-GCU-PHD-Z-09 Student of Doctor of Philosophy in the subject of Zoology session 2009-2012, hereby declares that the matter printed in the thesis tilted **"**The genomic basis and environment as risk factors for myocardial infarction" is my own work and has not been printed, published and submitted as research work, thesis or publication in any form in any university, Research institution etc in Pakistan or abroad.

_____ _____

Dated Riffat Iqbal

RESEARCH COMPLETION CERTIFICATE

It is certified that the research work contained in this thesis entitled **"The genomic basis and environment as risk factors for myocardial infarction"** has been carried out and completed by **Miss Riffat Iqbal** Roll No 16-GCU-PHD-Z-09 under my supervision

Dated

Supervisor
Prof. Dr. Nusrat Jahan

Prof. Dr. Nusrat Jahan Chairperson
Department Of Zoology
GC University Lahore

Controller of Examination
GC University Lahore

This dissertation is

Dedicated

To

my Beloved Parents,Husband

AND Kids

Who has been a source of love, mercy, sympathy and forgiveness

ABSTRACT

Myocardial Infarction (MI) is one of the major cardiovascular diseases worldwide. It is caused by rupture of atherosclerotic plaque in coronary vessels. Genetic and environmental factors play a key role in the development of MI. The major objective of current study was to evaluate the association of genetic variants with lipid metabolism and predisposition risk factors along with environmental factors in MI patients of Pakistani population. The study was designed in two phases; the first phase included demographic characteristics and economic burden of MI while the association of genetics with MI was investigated in the second phase. A total of 515 patients of MI were recruited to identify the economic burden, life style, family history and risk factors (hypertension, diabetes, smoking and hyperlipidemia) with MI. In second phase 384 Pakistani individuals were included for genetic analysis. A total of nine candidate genes with 22 SNPs were selected and genotyped by sequencing as well as one label extension method. The MI was significantly higher ($P < 0.05$) among males as compared to females in both urban and rural MI patients. The 43.11% patients were overweight (BMI > 25). The urban MI patients were significantly more overweight as compared to rural patients ($P < 0.05$). The 72.04% patients were found with previous family history of heart attack. Smoking (60.9%) and sedentary life style (70%) were more common in MI patients. Sedentary life style was predominant in Urban MI patients as compared to rural MI patients. The average annual cost per patient was found 9524.53 PKRs (96.96 USD). In genetic analysis 15 SNPs (out of 22) from 9 candidate genes were significantly ($P < 0.05$) associated with elevated risk of MI. Overall current study was the first to identify three novel SNPs rs10757278, rs10811656 and rs10757283 on chromosome 9p21.3 (*CDKN2A/B* gene) using 11 genetic markers, against MI in Pakistani population. The genetic variants rs10811656 risk allele T and rs10757278 risk allele G, rs10757283 risk allele T residing at chromosome 9p21.3 were found to be significantly associated with higher risk of MI [OR = 1.67 (1.22, 2.29), 1.37 (1.09, 1.72) and 1.47 (1.08, 2.01) respectively]. Two lipid metabolism related SNPs: rs662799 and rs3135506 of *APOA5*

were associated with risk of higher triglyceride levels (266 mg/dl genotype GG and 244 mg/dl genotype CC respectively) irrespective of age, gender, obesity, diabetes, hypertension and smoking. Four SNPs (rs2383206, rs2383207, rs10811656 and rs10757278) of *CDKN2A/B* (chromosome 9p21.3) were found in strong linkage disequilibrium [LD ($D^{/}$ = 0.99)] and their minor allele frequencies were significantly more prevalent in patients than controls (*P* = 0.02, 0.0002, 0.0012, 0.005 respectively). The four SNPs from 9p21.3 showed one risk haplotype (G-A-T-G; *P* = 0.001) and two protective haplotypes (A-G-C-A and G-G-C-A; *P* = 0.006, 0.001 respectively) involved in progression of MI. In addition the SNPs rs3135506, rs1558861, rs662799 and rs10750097 residing in *APOA5* gene were depicted strong linkage disequilibrium ($D^{/}$ = 0.99). Present study identified C-T-G-A and G-C-A-G haplotypes as risk haplotypes significantly (*P* = 0.0001) associated with MI. Higher BMI, smoking, hypertension, hyperlipidemia and diabetes were identified as strong predictor of MI in North Punjab Pakistan. Current study confirms correlation between lipid metabolism related SNPs and variants of 9p21.3 locus with MI as well as supporting the role of *APOA5* in raising the triglyceride levels. Preventive measures are needed to start at early age and continue throughout the life course. However further studies are needed for delineating the role of these SNPs in MI development.

ACKNOWLEDGEMENTS

All the praises are for Allah Almighty the creator of the universe and our beloved Prophet (SAW) for whom this wide and huge universe was assembled and who is the pioneer of education and research. Being his spiritual follower and believer, I tried to get educated and explore the artifacts and secrets of the universe.

I would like to thanks Prof. Dr Nusrat Jahan, Chairperson, Department of Zoology, Govt College University (GCU) Lahore, my thesis supervisor for her kindly guidance, invaluable support for my work throughout thesis academic years. Her wisdom and criticism has enlightened my way of logical thinking.

My special gratitude also goes to Prof. Dr Hong Xue, Director, Applied Genomic Centre, and Professor, Division of Life Sciences Hong Kong University of Science and Technology (HKUST) Hong Kong, for her helpful continuous encouragement, support, experience and close supervision throughout the work.

I sincerely acknowledge the effort, help, cooperation and valuable suggestion of Dr Atif Hanif (Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Lahore, Pakistan). He helped me a lot in learning research and also provided best possible facilities in his laboratories. A large depth of thankfulness to Yisuo Sun, Applied Genomic Centre and Division of life sciences, HKUST, Hong Kong for helping me to learn the experimental techniques and helping out tremendously with this task. I will never forget his help and concern.

I would express my appreciation and sincere gratitude to Dr. Ahmad Shahbaz (MBBS MS FACC Assistant Professor Cardiac surgery, incharge Department of Research, Training & Postgraduate Medical Education Punjab Institute of Cardiology, Lahore) for his guidance and help in sample collection for this study.

I am also grateful to Mr. Imran Haidar, Mr. Wadood alam, Mr. Asghar (office bearers) and Mr. Ali Imran (Store Keeper) Department of Zoology for their co-operation and provision of necessary chemicals and equipments during the course of research. Special thanks are direct to Miss Peggy WK Lee (the technician in laboratory at HKUST, Hong Kong), Wai Kin Mat (HKUST) for providing of material needed for research work.

I highly acknowledge Higher Education Commission of Pakistan for financial support for indigenous and foreign scholarship (Hong Kong University of Science and Technology) the completion of this worthy task.

I feel proud to mention the sincere efforts and co operation of my friends Andleeb Batool and Shaista Aslam for their continuous support, patience, encouragement and persuasion. In addition, I wish to express my thanks to my friends Irfana Liaqat, Sonia Tehseen and Najiya for their help during studies and research work. I really acknowledge the support, care and co-operation of my whole family who brought me up and raise their hands to complete my education and support me during my course of studies.

I am forever deeply grateful to my parents, husband, my kids Ahmad Bilal Ahsan and Maryam Eshal Ahsan for their infinite spontaneous joyful attitude to life. They always make me smile and give encouragement.

RIFFAT IQBAL

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVATIONS

ANRIL	Antisense non-coding RNA in the p16 locus
APOA5	Apolipoprotein A5
Bp	Base pair
CDKN2A	Cyclin dependent kinase inhibitor 2A
CELSR2	Cadherin EGF LAG seven-pass G-type receptor 2
CHD	Coronary heart disease
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FP	Florescence polarization
HDL-C	High density lipoprotein cholesterol
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase
HWE	Hardy Weinberg Equilibrium
LD	Linkage disequilibrium
LDL-C	Low density lipoprotein cholesterol
LDLR	Low density lipoproteins receptors
MI	Myocardial infarction
NCBI	National center for Biotechnology Information
NSTEMI	Non -St segment elevation myocardial infarction
NUTF2	Nuclear transport factor 2
OLE	One label extension
PCR	Polymerase chain reaction

PKRs	Pakistani rupees
PSRC1	Proline/serine-rich coiled-coil 1
rs number	Reference SNP number
S	SNP
SNP	Single nucleotide polymorphism
STEMI	St segment elevation myocardial infarction
Taq pol	Thermus aquatiqus polymerase
VLDL-C	Very low density lipoprotein cholesterol
WHO	World health organization

CHAPTER 1

INTRODUCTION

1.1 Myocardial infarction

Myocardial Infarction (MI) is a predominant and inevitable consequence of coronary heart disease (CHD) (Thygesen *et al.,* 2007). MI commonly known as heart attack occurs when thrombosis is precipitated by rupture of atherosclerotic plaque, it leads to hypoxia and subsequential necrosis of the myocardium (Alpert *et al*., 2000).

The prevalence of CHD in USA during the year 2010 was 19.8% among age group of ≥ 65 years as compared to age range of 45 to 64 years (7.1%). When data was compared with different ethnic groups, it was further noticed burden of CHD in year 2010 was higher among American Indians (11.6%) as compared to whites (5.8%) and Hispanics (6.1%) (Fang *et al*., 2011).

In 2005, globally total number of deaths by cardiac disease and stroke had been increased to 17.5 million comparing from14.4 million in 1990 (Go *et al*., 2013). Among these, 7.6 million deaths occurred only due to CHD (Dinc *et al*., 2013). CHD is the cause of more than 17.1 million deaths per year throughout the world (Chaudhry *et al.,* 2012) and it will be most serious leading cause of death worldwide by 2020 (Murray and Lopez, 1997). There were 39% deaths in low and middle income countries under the age of 70 years due to CHD (Gaziano *et al*., 2010).

Asian population is more susceptible to MI (Kearney *et al*., 2005; Joshi *et al.,* 2007). South Asia (India, Pakistan, Bangladesh, Nepal, and Sri Lanka) consists of more than quarter of developing world and was strongly affected by the increasing economic burden of CHD (Mc Keigue *et al.,* 1989). It had been depicted that South Asians suffer from first heart attack at the age of 53 years, which is six years earlier than European countries (Gaziano *et al*., 2010). MI was 50% higher in South Asians, which bear greatest burden of coronary risk as compared to white people in the UK (Gholapa *et al.*, 2011).

In North India, the prevalence of CHD had been reported 11% in 2001 (Mohan *et al*., 2001). Pakistan is a developing country with the population of 187 million, it accounts for alarming cardiac disease burden, estimated that 5.0937 million people were affected by cardiovascular disease in year 2006 (Yousaf, 2012). Moreover, the Pakistan accounts for 35% to 40% of cardiac disease burden (Wasif, 2011). One out of five individuals suffers with CHD in urban areas of Pakistan (Jafar *et al*., 2005). As stated by National Health Survey of Pakistan, mortality due to CHD is increasing day by day (Memon and Samad, 1999).

1.2 Types of MI

The experts of European Society of Cardiology (ESC), the American Heart Association (AHA), the American College of Cardiology Foundation (ACCF) and the World Heart Federation (WHF) proposed the third new universal definition of MI as the elevation of cardiac biomarkers in blood with any one biomarker greater than 99th percentile as Upper Reference Limit (URL). In addition presence of one of the following symptoms; Ischemia, Electrocardiograph (ECG) changes or thrombus diagnosed by angiography (Daubert and Jeremias, 2010). According to new definition MI is divided into following five types (Thygesen *et al.*, 2007).

- ST Segment Elevation Myocardial Infarction—STEMI—Type I
- Non-ST Segment Elevation Myocardial Infarction—NSTEMI—Type I
- Type II
- Type III
- Type IV and V

1.2.1 ST Segment Elevation Myocardial Infarction (STEMI)

The ST Segment Elevation Myocardial Infarction (STEMI) is a type of heart attack with damage over a large area of cardiac muscles (myocardium). In this type attack full thickness damage to myocardium take place. The ST-segment is a small portion of

ECG. It is characterized by the ECG changes in which elevation of ST segment shows complete thickness injury of myocardium (Van de Werf *et al*., 2008). Normal ECG is shown in Figure 1.1 (http://research.vet.upenn.edu).

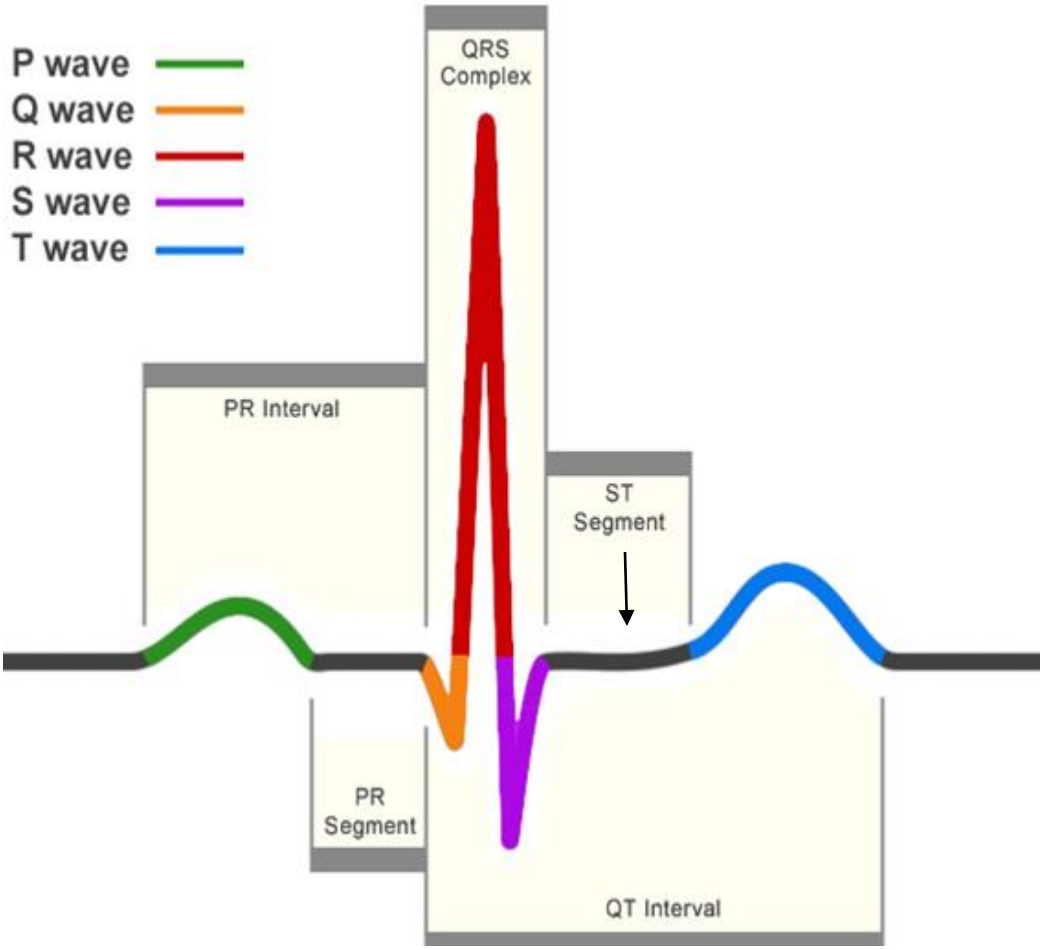


Figure 1.1: The waves of a single normal ECG pattern

1.2.2 Non- ST Segment Elevation Myocardial Infarction (NSTEMI)

Non ST Segment Elevation Myocardial Infarction (NSTEMI) is not acompained by ST elevation and usually is not full-thickness infarction (Figure 1.2). The NSTEMI is less severe in comparison with STEMI type of heart attack. The NSTEMI does not changes the pattern of ECG but it is diagnosed by the presence of raised myocardial biomarkers in the blood (Troponin, creatin kinase and myoglobin). Troponin found in cardiac muscle fibers and its concentration ≥ 0.01 ng/mL in the blood stream indicating damage to heart muscles (Van de Werf *et al*., 2008). Differnce of STEMI and NSTEMI is explained in Figure 1.3.

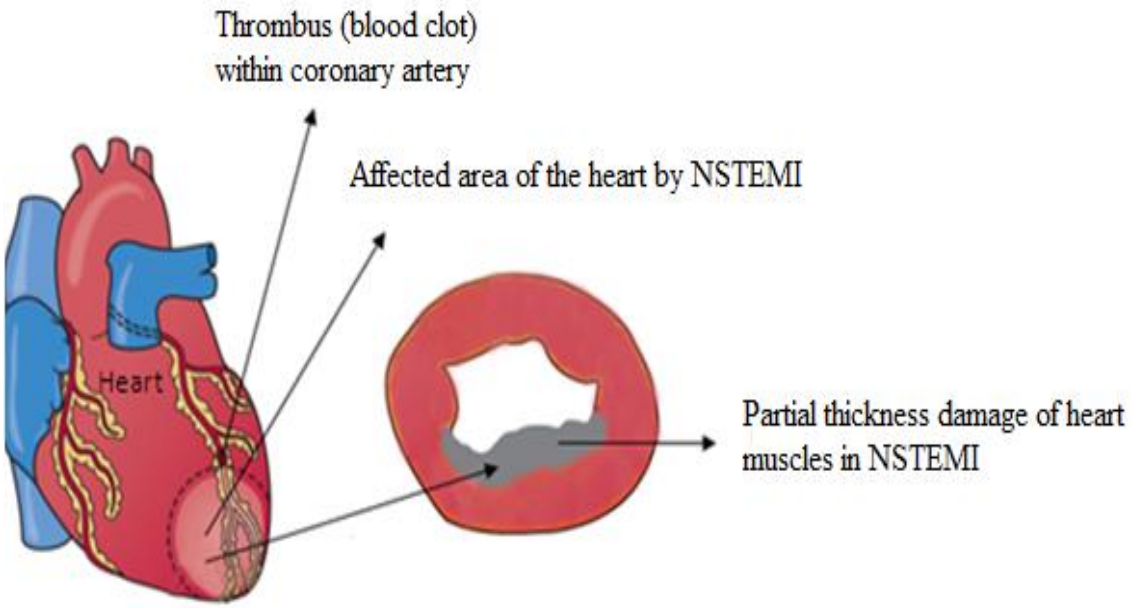


Figure 1.2: Partial damage to heart muscles in NSTEMI type of heart attack

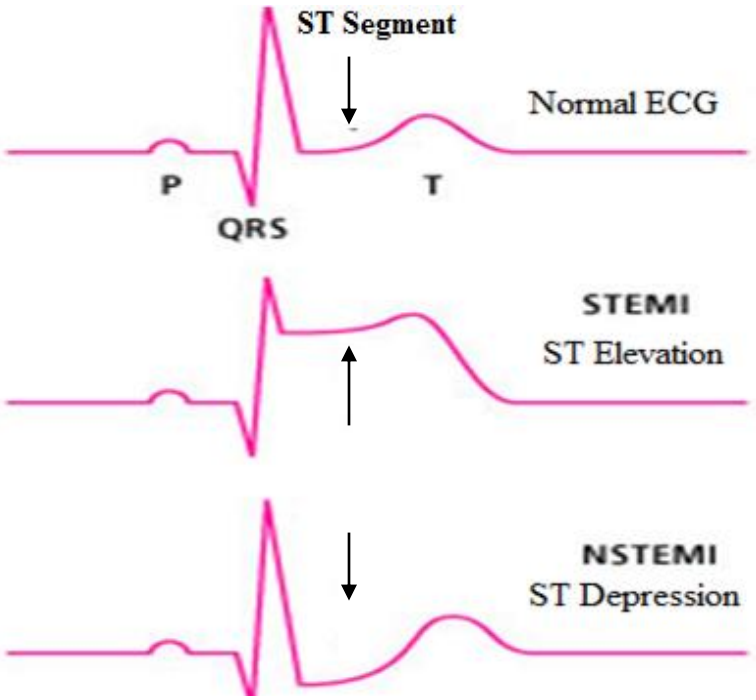


Figure 1.3: Difference between STEMI and NSTEMI

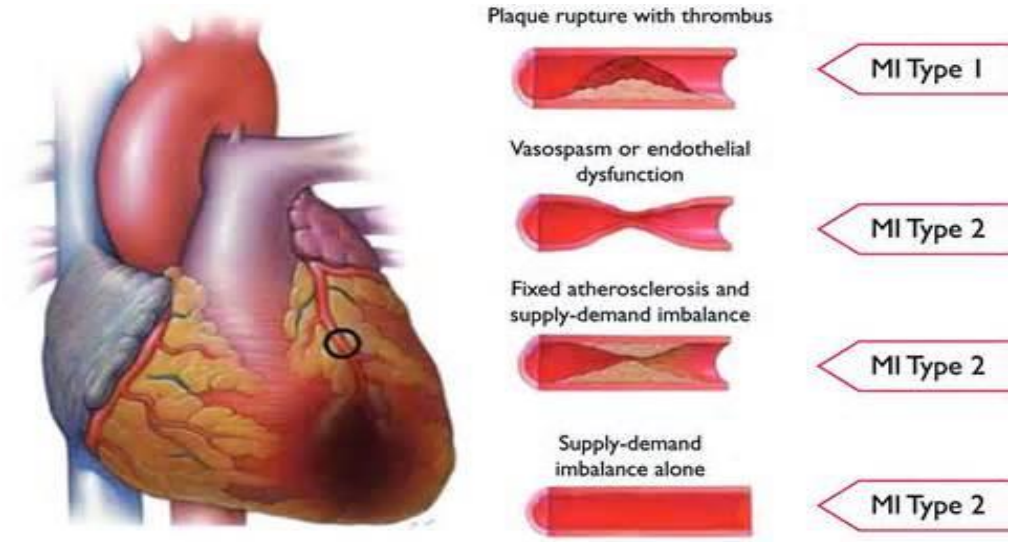
1.2.3 MI type 2 (Secondary to ischemia)

It is secondary to ischemia caused by imbalance between supply and demand of oxygen. MI type 2 is caused by hypotension, anemia, coronary embolism, spasm of coronary artery and dysfunction of coronary endothelium.

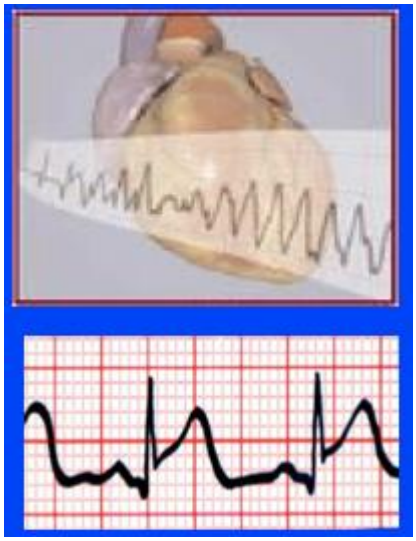
1.2.4 MI type 3 (Sudden infarction)

Type 3 is a sudden infarction by fresh thrombus appearance in coronary artery before emergence of biomarkers in blood (Figure 1.4)

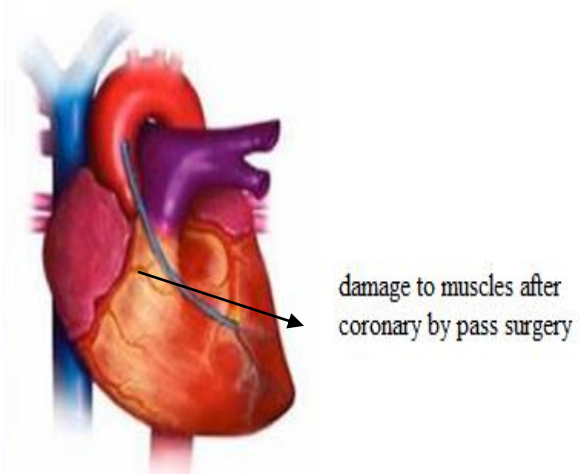
.



(a)



(b)



(c)

Figure 1.4: Differentiation between myocardial infarction according to the condition of the coronary arteries. (a) type 1 and 2 (b) type 3 (c) type 4 and 5 (Thygesen *et al.*, 2007).

1.2.5 MI type 4 and type 5 (Stent thrombosis)

Infarction related to stent thrombosis or coronary bypass surgery .

1.3 Pathophysiology

CHD is caused by atherosclerosis (the thickening and hardening of the inside walls of arteries). In atherosclerosis, plaque deposits develop in the arteries. Plaque is made up of fat, calcium, cholesterol and other substances from the blood (Thygesen *et al*., 2007). When a blood clot develops at the site of plaque in coronary artery then suddenly cuts off most of or all blood supply to that part of the heart muscles. If cells of myocardium do not receive enough oxygen they begin to die. This can cause the permanent damage to heart muscles. The process of plaque formation starts up in childhood, leads to young adults and appears with clinical symptoms in the middle age. It was demonstrated that endothelium injury can start the process of atherogenosis (Libby, 2001) (Figure 1.5).

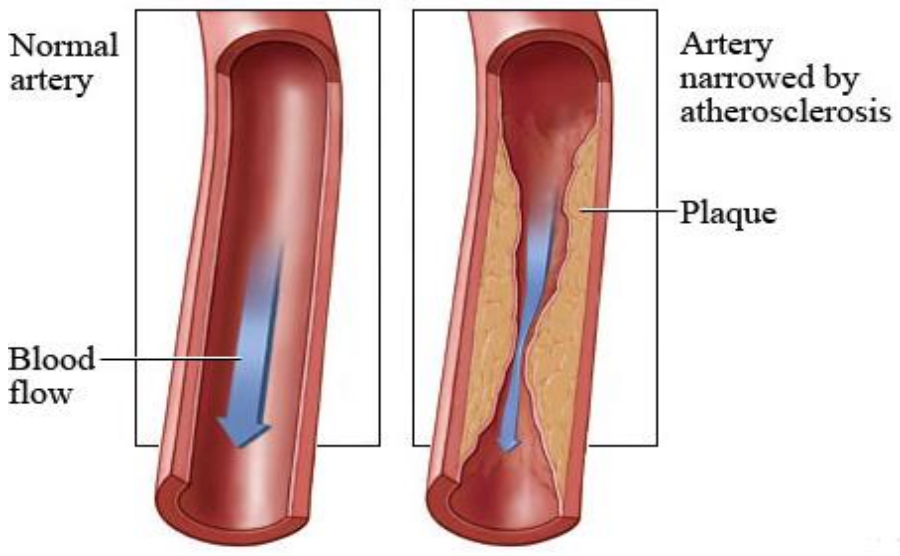


Figure 1.5: Narrowing of a normal coronary artery (Atherosclerosis) (www.webmd.com)

1.4 Symptoms

The World Health Organization (WHO) had defined MI, as presence of two or three following symptoms; chest pain/discomfort of 30 min duration, imbalance in cardiac enzymes such as troponin I, creatine kinase, lactate dehydrogenase isozymes specific for the heart, typical electrocardiogram pattern and shortness of breath (Capewell *et al.*, 2001).

1.5 Risk factors

Genetic and environmental factors have been studied as being responsible for high risk of MI (Wang *et al*., 2008; Topol *et al.*, 2006). Kannel. (2000) in his study has depicted six primary risk factors: age, obesity, hypertension, smoking, diabetes mellitus and hypercholesterolemia as major factors for the onset of CHD. Nuri *et al.* (2006) has demonstrated high prevalence of MI risk factors in Pakistan with more than 30% of the population affected by this disease over 45 years of age. Etiology of the CHD is not known, however the risk factors have strong statistical correlation with the onset of cardiac disease and its modification may lead to reduction in this disease (Goldstein *et al*., 2006).

1.5.1 Age/Gender

The high prevalence of cardiac disease at the age of 45 years in men and 55 years in women was reported in Pakistan (Ahmad and Shafiq, 2003; Jafar *et al.*, 2005). The male: female ratio of patients with coronary heart disease demonstrated as 3:1 along with gender age gap of 5 years (Jafar *et al.*, 2005). These gender differences in the onset of MI become narrow with increasing age. Risk of MI increased in postmenopausal women and those who used oral contraceptive pills (Dunn *et al.*, 2001).

1.5.2 BMI/ Obesity

Obesity is defined as the total body fat contents of more than 20% in average young males and over 30% in females. Body Mass Index (BMI) more than 25 kg/m^2 is

considered as overweight however, over 30 kg/m^2 is considered as obese (Edelstein and Sharlin, 2008). High BMI ($\geq$ 30 kg/m^2) has been independently associated with risk of MI at early age (Gupta *et al.*, 2003). More than 2.5 billion deaths are related with overweight worldwide (Brundtland, 2002). Asians have 3% to 5% higher body fat contents than whites at any given BMI (Prasad *et al.*, 2011). Fat contents cause glucose intolerance and increased diabetes in South Asian countries (Gupta *et al*., 2006). The prevalence of obesity in rural areas of Peshawar Pakistan was much higher among females as compared to males (Hassan *et al.*, 2005; Basit and Shera, 2008).

1.5.3 Hypertension

High blood pressure (hypertension) is one of the most important preventable cause of number of diseases worldwide (Lloyd-Sherlock *et al*., 2014). It has strong relationship with heart disease. In Pakistan, the magnitude of hypertension is very high (Nishtar, 2002; Dodani *et al*., 2004; Yusuf *et al*., 2004). It was reported that number of hypertensive patients will expand to 60% by 2025 worldwide (Kearney *et al*., 2005).

1.5.4 Diabetes

MI is the leading cause of death in diabetic patients and approximately 25 percent of heart attack survivors have diabetes (Fuuler *et al.*, 2001). It is a more potent risk factor in women than men since diabetic women have twice the risk of a second heart attack compared to diabetic men (Gale and Gillespie, 2001). South Asians have higher risk of type 2 diabetes (Nishtar, 2002), insulin resistance and bad fat distribution relative to other popultaions (Mc Keigue *et al*., 1989). It has been reported insulin resistance raised the risk of heart disease through inflammatory and lipid mediated mechanisms (Nishtar, 2002). Pakistan ranks at number six in terms of diabetic people worldwide (Wasay and Jabbar, 2009). Risk of MI increased in diabetic patients because impaired glucose level take part in progression of atherosclerosis and badly affect the cholesterol levels (Cade, 2008).

1.5.5 Cholesterol

Cholesterol is a steroid, used in the formation of cell walls, building of bile and hormones. A certain amount of cholesterol is necessary for good health but very high level in the blood is found to be associated with cardiac disease. Cholesterol enters the body from digestive system attached to the chylomicrons (lipoprotein particles) (Danik *et al*., 2008).

1.5.5.1 The cholesterol carriers

Cholesterol does not dissolve in the blood, it is conveyed by some protein packages, which facilitate its solubility. These cholesterol transporters are lipoproteins, known as Very Low Density Lipoproteins (VLDL), Low Density Lipoproteins Cholesterol (LDL-C), High Density Lipoproteins Cholesterol (HDL-C) and chylomicrons (Alberts *et al*., 2002).

VLDL is converted into LDL-C which is the bad cholesterol (Alberts *et al*., 2002). LDL-C is potent transporters of cholesterol from liver to blood. It accumulates fats into blood. High plasma LDL-C level (≥ 160 mg/dl) leading to the formation of atherosclerotic plaque (Miller *et al*., 2011). On the other hand HDL-C (good cholesterol) has opposite function and involved in transport of blood cholesterol to liver where it can be degraded and lower the blood cholesterol levels. High HDL-C provides the great protection from CHD. Triglycerides are blood fats, higher triglycerides (≥ 200 mg/dl) are also important risk factor for progression of MI (Danik *et al*., 2008). It has been estimated that high triglyceride concentration involved in increasing the blood viscosity, resulting in the slow blood flow. Viscous blood is more difficult to flow, so very minor amount of oxygen is delivered to body tissues and heart.

1.5.5.2 Hyperlipidemia

Hyperlipidemia stands as the third most important risk factor of CHD (Khan *et al*., 2005). It can cause blockage of coronary arteries and increase the risk of MI. INTERHEART study reported that abnormal lipid levels in blood are strongly associated with MI among South Asians (Chow *et al*., 2011). About 56% of heart diseases are

attributed to total serum cholesterol levels > 5.2 mmol/l or 200 mg/dl (Yusuf *et al.*, 2004). Hyperlipidemia accounts for 4.4 million deaths annually, which are 7.9% of the total global deaths and 2.8% of the total disease, burden worldwide (Shahid *et al.*, 2009). Increased levels of LDL-C and Low plasma HDL-C are associated with higher incidence of both atherosclerosis and MI (Vergeer *et al*., 2010).

1.5.6 Smoking

Smoking constitutes major factor in development of CHD. Even non-smokers, that inhaled smoke by any mean, also greatly increases MI risk (Whincup *et al.*, 2004). Cigarette smoke contains more than 4,000 chemicals, which has pathophysiological effect on heart. Smoking promotes plaque formation and participates in acceleration of atherosclerosis (Ambrose and Barua, 2004). Many hematological, metabolical and physiological changes associated with smoking. Due to smoking, heart demands more oxygen but the capacity of its supply to heart reduces. It results in increased oxidative stress which indicates a potent mechanism for initiating cardiovascular dysfunction. (Goldstein and Niaura, 2000). Shahid *et al.* (2009) reported cessation of smoking can reduce the risk of heart disease by 65%.

1.5.7 Life style

Pakistani children are acquiring unhealthy life style. Physical inactivity is constantly increasing among them (Khuwaja *et al*., 2003). Trends from physical activities i.e. active games and recreational sports have been changed to sedentary entertainment including extensive use of computer, television and video games. As a consequence Pakistani people develop CHD in their early age of 40 years (Gaziano *et al*., 2010). There is also an increasing trend of eating junk and empty caloric foods such as chocolates, snacks, and soft drinks. These habits have increased the obesity which itself is a potent risk factor for CHD, hypertension, diabetes and dyslipidemia (Vergeer *et al*., 2010). The risk factors associated with MI have been summarized in Figure 1.6.

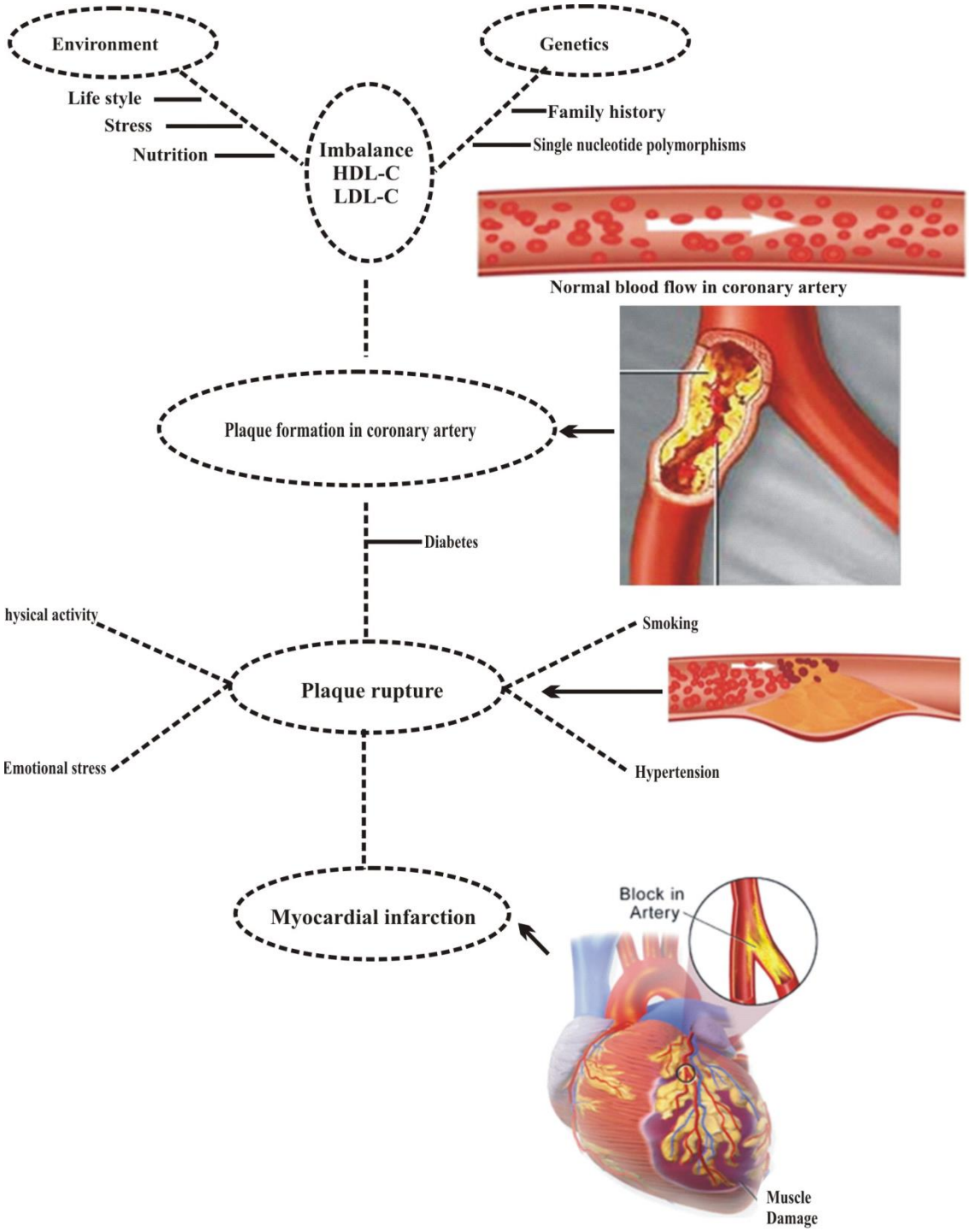


Figure 1.6: Schematic diagram showing myocardial infarction is a complex disease affected by environmental and genetic factors.

1.6 Genetics factors

MI is heritable and among the leading cause of disability and deaths worldwide (Rosamond *et al.,* 2008). It has been reported that if the first degree relative of a person is suffering from CHD the chances of that person getting the same problem increases manifold (Ryan *et al*., 1999). It is highly important for that person to make specific target oriented efforts to avoid the development of any other risk (Rosamond *et al.,* 2008). Genetic factors contributing to MI susceptibility also include common Single Nucleotide Polymorphisms (SNP).

1.7 Candidate and susceptible genes of MI

MI is polygenic trait inherited with positive family history. Genes associated with MI are found on many chromosomal regions; 9p21.3, 1p13.1, 2q36.3, 10q11.2 (Pasmant *et al.,* 2007; Kathiresan *et al.,* 2008; Mubotter *et al.,* 2012). The following genes were selected to evaluate the associations with cardiac disease.

1.7.1 *CDKN2A*

Cyclin dependent kinase inhibitor 2A and 2B are also known as p16 and p15 respectively. These proteins are encoded by *CDKN2A* and *CDKN2B* genes. These genes are located on chromosome 9 (9p21.3). The role of chromosome 9p21.3 locus in pathogenesis of CHD may be due to presence of strongly associated SNPs found about 100 kb from *CDKN2A* and *CDKN2B* genes (Samani *et al.*, 2007). These proteins play a role in regulation of cell cycle and mutation can leads to atherosclerosis (Schunkert *et al*., 2008). Function of these two inhibitors is to arrest the cell cycle at G1 and G2/S phases also block them from phosphorylation. So these genes are involved in playing an important role in cell aging, cell proliferation and apoptosis, leads to the formation of plaques inside arteries. Both genes were involved in the pathogenesis of atherosclerosis and MI (Pasmant *et al.,* 2007).

1.7.2 *ANRIL*

CDKN2B-AS, also called as *ANRIL* (antisense non-coding RNA in the p16 locus) is a lengthy non-coding RNA comprising of 19 exons, its spliced part is a 3834 bp RNA. It is found within the p16/*CDKN2A*- p15/*CDKN2B* gene cluster, in the antisense direction. SNPs in this region alter the expression of *ANRIL* are significantly associated with number of diseases, including diabetes, cardiac disease and cancer (Mubotter *et al.*, 2012). Genes associated with chromosome 9p21 are shown in Figure 1.7.

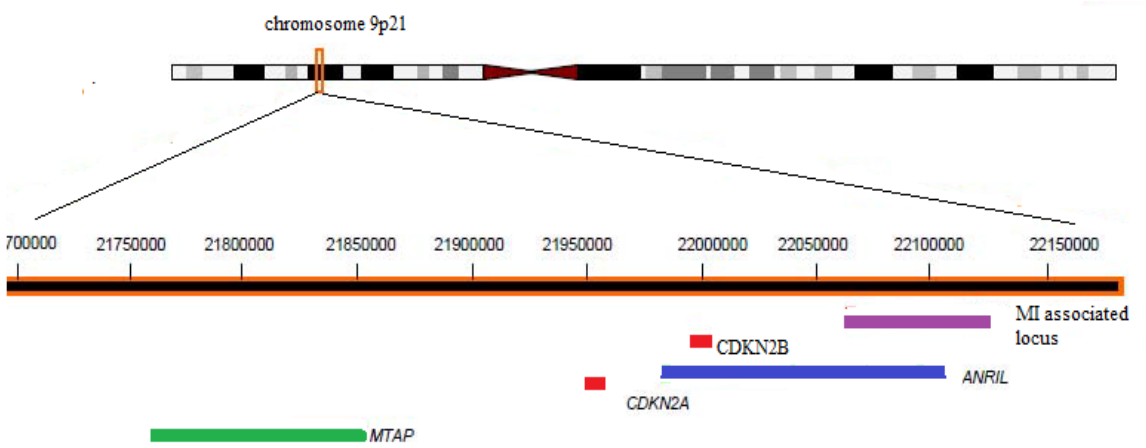


Figure 1.7: Chromosome 9, band p21 region. The location of the MI-associated region is shown in purple, annotated genes are shown in red and green, and the *ANRIL* anti-sense non-coding RNA is shown in blue. http://www.genecards.org/cgi-bin/carddisp.pl

1.7.3 *APOA5*

Apolipoprotein A5 is encoded by gene *APOA5*. This gene is found on chromosome 11 at long (q) arm on position 23 (11q23) (Figure 1.8). The *APOA5* gene consists of 4 exons. Its protein express in liver. The protein which is encoded by this gene regulates plasma triglyceride levels, a strong risk factor for cardiac disease. Apolipoprotein is associated with HDL-C and LDL-C. Mutation in this gene known to be associated with high triglyceride levels (Pennacchio *et al.*, 2001). Many genetic studies had suggested

that variation in circulating lipid levels is determined by lipid metabolism related SNPs (Garcia *et al.*, 2001; Samani *et al.*, 2007).

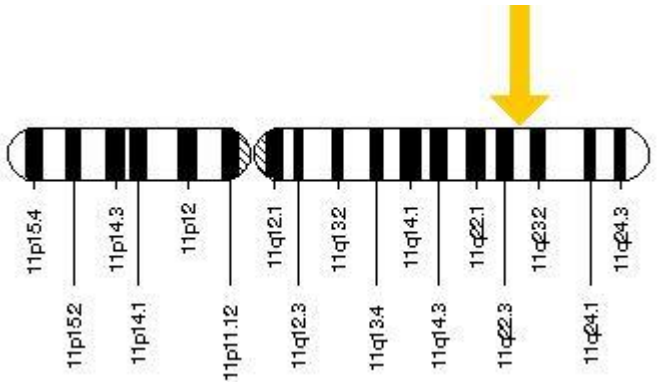


Figure 1.8: Chromosome 11, band q23 region. The location of the MI-associated region is shown in arrow. http://ghr.nlm.nih.gov/gene/APOA5

1.7.4 *PSRC1*

PSRC1 (proline/serine-rich coiled-coil 1) is a potent protein encoded by *PSRC1* gene. Its function is to regulate the mitosis by microtubule assembly. *PSRC1* gene is located on chromosome 1p13.3. Its protein consists of 63 amino acids. Hypertriglyceridemia is associated with mutation in this gene (Kooner *et al*., 2008).

1.7.5 *CELSR2*

Cadherin EGF LAG seven-pass G-type receptor 2 is also a protein of *CELSR2* gene. This gene is located on 1p21 locus. Its protein acts as receptor which involved in cell mediated communication, signaling of cells, protein localization and cell differenciation (Kathirersan *et al.*, 2008).

1.7.6 *HMGCR*

HMG-CoA reductase is the enzyme involved in cholesterol synthesis. This enzyme is suppressed by cholesterol derived from degradation of LDL through LDL

receptors. Inhibitor of this enzyme induces the expression of LDL receptors, which increases the catabolism of plasma cholesterol and potent factor for atherosclerosis. This enzyme play a central role in cholesterol biosynthesis and elevates the risk of MI. *HMGCR* gene which is located on 5q13.3 locus, SNPs in this region may imbalance the plasma LDL-C (Kathiresan *et al.,* 2009).

1.7.7 *NUTF2*

Nuclear transport factor 2 is protein encoded by *NUTF2* gene. This protein facilitates transport of ions, macromolecules and small molecules in to the nucleus. *NUTF2* gene is located on chromosome16q22.1 (Willer *et al*., 2008).

1.7.8 *LDLR*

Low density lipoproteins (LDLR) are receptors that involved in the uptake of LDL particles. These receptors play an important role in plasma lipoprotein balance. Genetic polymorphism in *LDLR* gene (SNPs) can alter its expression and participate to lipoprotein imbalance (Gao *et al*., 2013).

1.8 Single Nucleotide Polymorphisms (SNPs)

SNPs are single base pair variations within the genome which are present at more than 1% frequency in human population (Risch, 2000). Variation of a single nucleotide A/C/G/T can alter the sequence of gene. It has been identified more than 1.4 million SNPs found in the human genome (Lander *et al.*, 2001). Variants may be found throughout whole genome e.g. in introns, exons, promoter (Tokuhiro *et al.,* 2003), intragenic regions and splicing sites (Betticher *et al.,* 1995). Some SNPs do not occur in coding region of genes. A SNP that is found in coding region can directly affect the relevant protein by changing the amino acid (Livingston *et al.,* 2004). However, SNP in promoter region may also affect the gene expression (Drazen *et al.*, 1999). Many multifactor diseases such as diabetes, cancer, mental illness and heart disease are influenced by SNPs pattern in associated gene (Alfredo *et al.,* 2007). The genetic polymorphisms may speculate an individual to be more susceptible to plaque formation,

increased lipid levels and thrombus formation (Wung, 2002). Risk factors for disease onset are influenced by genetic variants which could act by themselves or in combination with other lifestyle or genetic factors. Genetic variants that influence these risk factors may also be associated with onset of MI (Kathirersan *et al.*, 2008).

1.8.1 Haplotypes

Collection of SNPs along a chromosome is known as haplotype. Alleles that are found close to each other in a chromosome incline to be inherited together more than expected by chance. This phenomenon is known as Linkage Disequilibrium (LD). Polymorphisms which are in strong LD could not be getting apart even by recombination or mutation and thus transfer together in the next generation (Gabriel *et al.,* 2002). Haplotype based association analysis have LD information from multiple markers, thus are powerful tool for gene mapping (Zaykin *et al*., 2002).

1.9 Case-control association study

Genetic variants of complex diseases can be determined by association studies and linkage analysis (Daly and Day, 2001). Linkage analysis describes that region of genome with particular disease inherited within families (Bush and Moore, 2012). While in case control association studies the significant difference in allele and genotype frequencies between patient and control are taken as evidence for the involvement of allele in causing disease. There are many advantages of using case control association pattern of studies; cases and control are easy to enroll for sampling than family based, late progression of diseases can be studied, very large number of samples can be recruited for study and unreleated control increase strength of study for genetically related individuals (Daly *et al*., 2001; Cardon and Palmer, 2003).

1.10 Economic burden of MI

Majority of South Asians live on or below the poverty line with lack of healthcare systems or services, absence of National Welfare Programs and no provision of health insurance for this population (Jha, 2011). The poor people cannot afford to pay for

healthcare services. Cost greatly influences complication of CHD (Suhrcke *et al*., 2006). The low and middle income people are facing the socioeconomic burden to get their treatments (Nugent, 2008). Expenses of diagnosing the heart diseases through ECG, blood biomarkers, angiography, Computed Tomography (CT) scan and echocardiograph, etc. are very high. While, the main economic burden of cardiac disease is faced by a person at the time of heart surgery. The treatment of heart disease is a continuous process till end of their lives; patients have to consult their physicians every month, monthly medicine as well consultant charges are also difficult to afford (Zagrosek *et al*., 2011). When analysis of health expenditure was compared with household income in Pakistan depicted that ideal treatment is unaffordable for average patients (Nishtar, 2002). It has been reported burden of CHD is shifted towards the low socioeconomic strata in Pakistan (Gupta and Verma, 2006). One- third of Pakistani population is living in poverty; they cannot afford the increasing burden of such costly disease. Cost of treatment and diagnostic tests are very high and not affordable by poor people (Jones *et al*., 2009) Previous study of Pakistani population reported that treatment cost of heart disease runs into millions (Gaziano *et al*., 2010).

1.11 Aims and objectives

Current study evaluated SNPs in associated genes that may play significant role in the onset of MI. Identification of genes responsible for susceptibility to MI will be helpful for understanding pathophysiology of the disease, in selection of suitable treatment and gene therapy. In addition SNP database will also be helpful for early diagnosis and detection of the disease.

The major objectives are as follows:

- To study demographic characterisitics of MI in different districts of North Punjab, for the better management of disease.
- To evaluate the SNPs in candidate genes that may play significant role in the onset of MI.

CHAPTER 2

LITERATURE REVIEW

Cardiac disease was reported as leading cause of worldwide mortality (Gupta *et al*., 2008). The European Society and American College of Cardiology has defined MI (Christenson and Christenson, 2013) as cell death of myocardium which can be detected by the imbalance in biochemical markers (troponins) or pattern of ECG changes. It was reported that almost 1.5 million people suffer from MI annually in the United States (Siddiuqi and Kayani, 2000; Eyre *et al*., 2004). The Pathophysiological triggering mechanisms for the onset of MI has been reported from last few decades (Sari *et al*., 2009). The prevalence of heart disease increased with the advance age and differs within geographic, racial, socio demographic and ethnic groups (Rebecca *et al*., 2005).

High risk of MI had been demonstrated in South Asian population and was reported the leading cause of deaths in developing countries including Pakistan (Chadha *et al.,* 1990; Hafizullah *et al.,* 2010). It was revealed that deaths due to MI in South Asian countries were 5-10 years earlier than other regions of the world (Yusuf *et al*., 2004) while, high risk of MI in younger age was determined by the levels of related risk factors (Enas and Senthilkumar, 2001). Different aspects of cardiac disease studied in 52 countries among Asia, South America, Europe and Middle East found to be associated with various risk factors such as smoking, hypertension, history of diabetes, stress, alcohol intake, and physical inactivity (Roeters *et al*., 2002; Yusuf *et al*., 2004). The knowledge of simple conventional risk factors are not enough because of early occurrence of heart disease among South Asians therefore it was suggested both genetic as well as non conventional risk factors may play important role in the onset of disease at an early age (Ezzati *et al*., 2003).

2.1 Risk factors associated with MI

The prevalence of MI risk factors in many countries reached on peak levels. Mackay and Mensah. (2004) depicted the global percentage of risk factors involved in the

onset of CHD was as follows; smoking (12%), obesity (7%), hypertension (9%) and dyslipidemia (7%). In addition 75% of the cardiac disease burden implying that heart patients have one or two of these risk factors. The strength of risk factors with disease susceptibility varies between populations.

2.1.1 Age

It has been reported 29% MI cases were found at the age of 80 years in developed countries (Reikvam and Hagen, 2002). On the other hand in developing countries mean age of MI patients range from 55-60 years (Bogousslavsky, 2001). The young population with MI at the age of 53 years was also found in South Asian population (Yusuf *et al*., 2004).

2.1.2 Obesity

Obesity is major health problem worldwide (Bray and Bouchard, 2003). It is associated with heart disease (Poirier *et al*., 2006). It was reported that 21% cardiac diseases were due to obesity globally (Lavie *et al*., 2009). Average BMI in people of Brazil was recorded 25–26 kg/m^2 for females and 23-24 kg/m^2 for males (Anjos *et al*., 2013) while in Canada and USA above 27 kg/m^2 was recorded for both females and males (Reaven and Laws, 1999). The 19.8% US people of young age were obese (Mokdad *et al*., 2002) whereas, 13.2% males and 22.6% females were overweight in Pakistan (Pappas *et al*., 2001; Nanan, 2002; Afridi and Khan, 2004). National representative survey of Pakistan has reported 25% of Pakistani people to be overweight and 10.3% were obese (Humayun *et al.*, 2009). Obesity increases strain on the heart, raises blood pressure, cholesterol and increases diabetic risk (Eckel, 1997).

2.1.3 Hypertension

Van den Hoogen *et al.* (2000) investigated connection of heart disease with high blood pressure in six different populations (Japan, Greece, Italy, Finland, Yugoslavia and Netherland) around the world and found greatly increase in mortality of cardiac disease due to hypertension. The worldwide prevalence of hypertension was 972 million (26.4%) in year 2000, of which two out of three belongs to developing countries (Kearney *et al*.,

2005; Sultana *et al*., 2010). According to National Health Survey of Pakistan 1990-1994 the prevalence of hypertension in Urban areas was 21.5% (one in every three persons of age of 45 years) and only less than 3% of hypertensive patients had their blood pressure around normal range of 140/90 mmHg (Aziz *et al*., 2005). However, 43.68% cardiac disease in Pakistan was due to hypertension (Khan *et al*., 2005). In Pakistan estimated average systolic blood pressure of people with 30 years of age was 130-140 mm Hg (Syed *et al*., 2008) whiles in India 120-130 mmHg (Adler *et al*., 2000) and 120 mm Hg in Thiland (Mackay and Mensah, 2004). It was also reported that people with high blood pressure have early MI symptoms due to hypertrophy which is associated with hypertension (Rosendorff *et al*., 2007).

2.1.4 Diabetes

Diabetes mellitus and glucose intolerance are major risk factors for CHD in Asia (Steinberger and Daniel, 2003). Prevalence of diabetes has been increasing throughout Asia (Ramachandran *et al*., 2008). Risk of diabetes varies between populations from 7.2% among Australians to 21% among younger Arabians (Dunstan *et al*., 2002; Malik *et al*., 2005). It was reported the prevalence of diabetes in peoples with aged ≥ 27 years was 5-5.9% in Pakistan and India, 15% in Qatar, 14.9% in Greece and < 5% in China (Mackay and Mensah, 2004). In Libya 48.2% hospitalize MI patients were diagnosed with diabetes (Abduelkarem *et al*., 2012). Furthermore, in Britain 10.5 - 30.0% MI patients were diabetics (Wilkinson *et al*., 1996; Alnemer *et al*., 2012).

The prevalence of type 2 diabetes mellitis in cardiovascular patients in Peshawar, Pakistan was reported 23.00% (Khan *et al*., 2005). INTERHEART study among 52 countries reported 18% diabetes cases among MI patients (Yusuf *et al*., 2004). In the terms of absolute number of people with diabetes; Pakistan, India and Bangladesh were three of top ten countries globally (Wild *et al*., 2004). South Asians have higher prevalence of type 2 diabetes and early onset of heart disease was reported despite of normal BMI in diabetic patients (Diaz *et al*., 2007; Joshi *et al*., 2007). Diseases, type 2 diabetes and cardiac are interlinked (Enas and Mehta, 1995). Diabetes had been reported

to be associated with left ventricular dysfunction and take part in progression of coronary atherosclerosis (Wilkinson *et al*., 1996).

2.1.5 Cholesterol

High cholesterol in serum was reported to be critical risk factor for CHD in Asian population (Okamura *et al.,* 2003). The recommended level of total cholesterol for average healthy individual in Asians is ≤ 240 mg/dl, where LDL-C level should be ≤ 160 mg/dl and HDL-C level should be 40 mg/dl (Battinelli, 2007). Abnormal lipid parameters (LDL-C, HDL-C and triglyceride) are powerful predictors of atherosclerosis and leads to heart attack (Ballantyne *et al*., 2001; Gabriel *et al*., 2008; Sigdel *et al*., 2012). The risk of heart disease increases significantly by 1 mmol/L (40 mg/dl) rise in total plasma cholesterol (Critchley *et al*., 2004). The mean plasma cholesterol level 117 mg/dl (6.5 mmol/L) was reported in Chinese people (Wu *et al.*, 2004). People of many countries had higher cholesterol even greater than 6.5 mmol/ L (Jousilahti *et al*., 1998), such as mean plasma cholesterol level 208 mg/dl (11.5 mmol/L) was recorded in America (Metz *et al*., 2000). Both LDL-C and HDL-C have antagonistic association with MI risk where HDL-C negatively and LDL-C being positively associated with MI (Di Angelantonio *et al*., 2009).

South Asians have higher levels of LDL-C and lower HDL-C levels as compared to other populations (Kulkarni *et al.,* 1999; Bhalodkar *et al.,* 2004). Lipid abnormalities were found especially in Pakistan and Bangladesh (Bhopal *et al.*, 1999; France *et al*., 2003). It has been recommended less fat intake by diet can reduce total blood cholesterol (Hooper *et al*., 2012). Both genetic and environmental factors are involved in hypercholesterolemia (Bhatnagar *et al*., 2008). The West of Scotland Coronary Prevention Study (WOSCOPS) found lowering cholesterol levels can reduce the risk of heart disease in those peoples having LDL-C concentration greater than 73 mg/dl (Rosamond *et al*., 1998).

Triglycerides had shown to play a key role in pathogenesis of MI (Hooper *et al*., 2012). Its level in plasma more than 150mg/dl is regarded as high (Sigdel *et al*., 2012). It had been found triglyceride rich lipoproteins are the cause of endothelial dysfunction by

entering into atherosclerotic plaques (Rapp *et al*., 1994; Carantoni *et al*., 1997) and also stop the cholesterol transport into liver (Palmer *et al*., 2004). The products of lipid peroxidation were found to be involved in plaque disruption (Hodis *et al*., 1994).

2.1.6 Smoking

There is a relationship between smoking and high cholesterol levels; this may reflect the increased intake of cholesterol with diet among smokers (Hebert and Kabat, 1990). Smoking as a very potent risk factor associated with dysfunction of endothelium and contributes to the onset of MI even in those individuals with minimal atherosclerosis (Yusuf *et al*., 2004). The incidence of smoking have been decreasing in European countries (Fuster and Kelly, 2010) however, has not been the case in Asian population where 40 - 60% incidence of smoking was recorded (Yang *et al*., 1999). Highest smoking rate in Chinese and South Asians (Hermalin and Lowry, 2011) was reported whereas, smoking was less common in South Americans (Li *et al*., 2007). The 62.6% people were found smokers in Libyan population in the year 2012 (Abduelkarem *et al*., 2012). It was demonstrated, if current trend of smoking persist will cause deaths of 8 million people worldwide each year by 2030 and of 80% deaths will be from developing countries (Kreatsoulas and Anand, 2010). Different studies in China (Loke *et al*., 1997), Brazil (Levy and Thom, 1998), Lebnan (Monteiro *et al*., 2004) and India (Malhotra *et al*., 2003) have reported 40% of patients with MI were smokers for most of the time in their lives.

According to local study estimates the prevalence of smoking in Pakistani individuals aged ≥ 8 years was 14.2% (95% CI: 13.6-14.8) and 19.4% (95% CI: 19.08-19.72) among group aged ≥ 15 years (Nasir and Rehan, 2001). The smoking was much prevalent among urban areas (15.2%) than rural areas (13.7%) of Pakistan (Alam *et al*., 2008). Smoking badly affects the lining of arteries which can double the risk of heart attack (Mehta and Eagle, 1998).

2.1.7 Hyperhomocysteinemia

Mild hyperhomocysteinemia (homocysteine level in plasma between I5-25 umol/L) is another factor which has positive association with heart disease (Donner *et al.*,

1998; Iqbal *et al*., 2006). Higher homocystein is a potent risk factor for atherosclerosis which leads to heart attack (Mc Cully, 2007). The nutritional deficiency of folate and vitamin B6 is the major cause of hyperhomocysteinemia in the Pakistani population (Iqbal *et al*., 2006; Yakub *et al*., 2010). The inadequacy of these vitamins in Pakistan may be due to minimum use of fresh fruits and vegetables, overcooking of food and high prevalence of parasitic enteric infections (especially amoebiasis and giardiasis) (Laghari *et al.,* 2010).

2.2 Knowledge of risk factors

The level of knowledge for MI varies among populations. It had been found US whites have high knowledge about onset and consequences of the disease (Ford and Jones, 1991). However, in South Asian families who inhabit in UK had lower awareness about cholesterol and dietary contents than native white people (Rankin and Bhopal, 2001). The less education is major factor for lack of knowledge among South Asians (Kandula *et al*., 2010). Less than 20% individuals have knowledge about risk factors of MI and its consequences in Pakistan (Dodani *et al*., 2004).

2.3 Treatment cost

Coronary heart disease is one of the costly diseases showing a huge economic burden on health care system. It had been reported in USA direct and indirect expenditure of heart disease in 1995 was 17,532 US$ annually/patient (Russell *et al*., 1998). Tarride *et al.* (2009) investigated treatment cost in eight different countries ranged from US$ 9512 (Belgium) to 18293 US$ (Austria), while in USA treatment cost as high as 32,975 US$/year/patient. The cost of MI treatment was 10 to 20 times greater than angina pectoris (Stewart *et al*., 2003). In Pakistan 75% people living in poverty are unable to afford the high expenditure (diagnosis, treatment, surgery) of such costly disease. Sehrish. (2011) demonstrated even diagnostic tests of cardiac disease in Pakistan are in the range of 40,000 to 50,000 PKR (405.3-506.7 US$/ patient).

2.4 Genetic factors

Several genes and genetic loci had been reported to showing association with onset of MI (Helgadottir *et al*., 2007; Saxena *et al*., 2007; Shen *et al*., 2008). Total eight Genome Wide Association Studies (GWAS) have been published for risk factors association at $P \leq 10^{-5}$ with CHD or MI. The remainder genetic studies have examined markers associated with CHD including HDL-C, LDL-C, C reactive proteins and coronary artery calcification **(**Mc Pherson *et al*., 2007; Kathiresan *et al*., 2009; Samani *et al*., 2009).

2.4.1 Genetic studies of 9p21.3 locus and related SNPs

Genetic variants of 9p21.3 region have great importance in molecular genetics consisting of; *CDKN2A, CDKN2B* and *ANRIL* genes (Samani *et al*., 2007. The risk haplotypes at 9p21.3 locus [(rs10757274-G), (rs4977574-G), (rs2891168-G), (rs2383206-G), (rs2383207-G), (rs1333049-C)] were shown to be overlaped with *ANRIL* gene (Broadbent *et al*., 2008).

GWAS have depicted strong association of chromosome 9p21 with susceptibility of MI (Helgadottir *et al*., 2007). Two SNPs in chromosome 9p21 locus (rs10757274 and rs2383206) were strongly associated with MI in Canadian cohort as well as five white cohorts (Mc Pherson *et al*., 2007). Two other SNPs at the same locus; rs10757278 and rs2383207 were studied in Iceland, German and British populations (Helgadottir *et al*., 2007; Samani *et al*., 2007; Yamada *et al*., 2008). Many independent case-control association studies have pointed out significant role of chromosome 9p21.3 locus with onset of MI in Sweden (Saxena *et al*., 2007), Finland (Scott *et al*., 2007), UK (Zeggini *et al*., 2007) and Italian population (Shen *et al*., 2008). Another variant rs1333049 (risk allele C) was demonstrated playing a key role in the formation of atherosclerotic plaque (Ye *et al*., 2008) and associated with onset of cardiac disease (Schunkert *et al*., 2008). Polymorphism at 9p21.3 locus was not associated with onset of MI in African Americans (Dehghan *et al*., 2008). The opposite results were may be due to ethnicity difference (Horne *et al*., 2008).

2.4.2 Genetic studies of other loci related to cardiac disease

Some variants at chromosome 1p13 (*CELSR2*, *PSRC1* and *SORTI*) were found to be associated with early onset of MI (Samani *et al*., 2007; Wallace *et al*., 2008; Kathiresan *et al*., 2009). *SORTI* gene had been implicated playing a key role in insulin resistance (Kaddai *et al*., 2009). In addition to this locus Lipoprotein Lipase (*LPL*) gene at 8p21 locus has also been replicated in multiple GWAS was associated with HDL-C and triglyceride levels (Kathiresan *et al*., 2008; Willer *et al*., 2008). Chromosomes 6q24 (rs12526453) and 10q11 (rs1746048) were investigated to be linked with MI (Huang *et al*., 2013).

Mutation in Low-density lipoprotein receptor (*LDLR*) and protein convertase subtilisin/kexin type 9 (*PCSK9*) at 19p13 involved in recycling of LDL receptors, leading to autosomal dominant hypercholesterolemia (Kathiresan *et al*., 2009). Genetic variants (rs2569556, rs12084215, rs565436 and rs23862269) in *LDLR* and *PSK9* regions have been shown to be significantly associated with plasma higher LDL-C level in Malaysia (Lye *et al*., 2013). Haplotype analysis of these genetic loci reported significant association of higher LDL-C levels with MI (Bostom *et al*., 1996).

Apolipoprotein E (*APOE*) gene is important predictor for LDL-C transport and metabolism, so responsible for removal of cholesterol from the blood (Lahoz *et al*., 2001). SNPs associated with *APOE* affect the cholesterol metabolism, which in turn play a potential role in onset of heart disease particularly heart attack. It has been depicted polymorphism in *APOE* gene was associated with higher risk of MI (Wilson *et al*., 1994; Lahoz *et al*., 2001).

Methylenetetrahydrofolate reductase (MTHFR) is an enzyme encoded by gene *MTHFR* has a key role in conversion of hemocystein (toxic amino acid) in to methionine. Mutation in *MTHFR* can cause plasma higher hemocyctein level associated with increased risk of MI (Christen *et al*., 2000). Genetic variant rs1801133 (677 C-T) of *MTHFR* gene (chromosome 1) had been depicted to be associated with reduction of *MTHFR* activity (Ueland and Rozen, 2005). Polymorphism of 677 C-T was associated with disease susceptibility (Gallagher *et al*., 1996; Kluijtmans *et al*., 1996; Helgadottir *et*

al., 2006). A meta-analysis from 40 studies showing; polymorphism of SNP rs1801133 in *MTHFR* gene with genotype TT had an odd ratio of 1.16 as compared to CC genotype (Klerk *et al*., 2002). Another meta-analysis from 80 studies showed variant in *MTHFR* gene associated with high risk of MI with an overall odd ratio of 1.14 for TT genotype, in addition odd ratio for population of Australia, Europe and North America was 1.0, whereas for Asia was 1.23. These results demonstrated 677C-T polymorphism is associated with risk of MI in Asian population, but not in Australia, North America and Europe (Lewis *et al*., 2005). Genetic regions reported to be associated with onset of MI or related phenotypes in GWAS are shown in Table 2.1.

Table 2.1: Overview of the genes associated with myocardial infarction or coronary artery disease that have been identified by GWAS

Chrom segment	Gene	Function	SNP	Population	Reference
1p13.3	*PSRC1, CELSR2, SORT1, MYBHL*	LDL-C increase	rs599839	Caucasian UK China Finland/Sweden/France Germany American Caucasians	Samani *et al*., 2007 Sandhu *et al*., 2008 Huang *et al.*, 2008 Karvanen *et al*., 2009 Kleber *et al.*, 2010 Wang *et al.*, 2011
1q41	*MIA3*	Collagen procession	rs300861 rs17465637	Caucasian American Caucasian	Samani *et al*., 2007 Wang *et al*., 2011
5q13.3	*HMGCR*	Cholesterol biosynthetic pathway	rs3846663	Americans Kosre	Chen *et al.*, 2009 Lowe *et al*., 2009

Table 2.1 continue

Chromosome segment	Gene	Function	SNP	Population	Reference
11q23	*APOA5*	Triglyceride regulation	rs1558861 rs10750097 rs3135506 rs662799 rs662799 rs651821	Europe China Italy Pakistan Hong Kong India	Wang *et al.*, 2008 Willer *et al*., 2008 De Caterina *et al*., 2011 Saleheen *et al.*,2010; Jiang *et al.*, 2010 Li *et al*., 2007; Liu *et al*., 2010
14q13	*PSMA6*	Inflammatory pathway	rs2277459 rs17458312 rs1048990 rs2277461	Japan UK China East Asia	Ozaki *et al*., 2006 Bennett *et al*., 2008 Liu *et al*., 2009 Wang *et al*., 2011
20q11	*MMP9*	Breakdown of extracellular matrix, polymorphism results in inflammation of aorta	rs3918242 rs2664538	Europe Turkish	Horne *et al*., 2008; Willer *et al*., 2008 Alp *et al*., 2009

Table 2.1 continue

Chromosome segment	Gene	Function	SNP	Population	Reference
9p21.3	*CDKN2A*, *CDKN2B*, *ANRIL*,		rs4977574 rs1333049 rs10757283 rs2891168 rs10757278 rs2383206 rs2383207 rs10757274 rs10811656	Iceland South Korea Caucasian British Italy Asian Indian Pakistan China America, Caucasians USA, Sweden, Finland, Spain, and Italy Finland and Sweden South Asia	Helogodottir *et al*., 2006 Shen *et al.,* 2007 Samani *et al*., 2007 Burton *et al.,* 2007 Shen *et al*., 2008 Maitra *et al.,* 2008 Saleheen *et al*., 2010 Wang *et al*., 2011 Abdullah *et al.,* 2008 Kathirersan *et al.,* 2008 Ripatti *et al.,* 2010 Peden et al., 2011

Table 2.1 continue

Chromosome segment	Gene	Function	SNP	Population	Reference
6q24	*PHACTR1*	Coronary calcification	rs12526453 rs9349379	USA, Sweden, Finland, Spain, and Italy. Europe	Kathiresan *et al.*, 2009 Schunkert *et al.*, 2011
6q26	*LPAL2*, *LPA*, *SLC22A3*	It encode protein which play role in lipoproteins pathway	rs2048327 rs3127599 rs7767084 rs10755578	Northern Europe China	Tregouet *et al.*, 2009 Zhang *et al.*, 2013
19p13	*LDLR*	Regulate LDL-C levels	rs1122608	USA, Sweden, Finland, Spain, and Italy Europe	Kathiresan *et al.*, 2009 Ripatti *et al.*, 2010

CHAPTER 3

MATERIALS AND METHODS

The study was conducted in two phases, in first demographic characteristics and economic burden of MI were studied. In second phase the association of genetics with MI was determined.

3.1 First phase

3.1.1 Data Collection

In this phase of study data was collected from six regions of north Punjab, Pakistan. Urban MI patients from developed areas (Lahore, Islamabad) and rural cases of developing areas (Faisalabad, Gujranwala, Gujrat and Sialkot) were included in this survey. The diagnostic criterioa (prescribed by the doctor or clinically diagnosed) was based on severe chest pain of 30 minutes duration, characteristic electrocardiographic patterns of MI and significant elevation of cardiac enzymes such as Creatine Kinase Myocardial Band (CK-MB). The study protocol was approved by ethical committee Board of Advanced Studies and Research Board (BASR). Participation rate of patients was 65%. Excluded subjects were 285 out of 800 because of incomplete data of variables. Thus 515 patients were included in this phase of study. Inclusion criteria were patients with MI as principal diagnosis and admission via CCU and emergency wards. A questionnaire was designed to collect data (Annexure I). The analysis was focused on identifying the socioeconomic status (education, employment status, income), life style (smoking, physical activity and dietary pattern), family history of MI and risk factors (diabetes, hypertension and hyperlipidemia) were obtained. All the subjects were assessed for blood pressure and body mass index [weight divided by square of height in meters (kg/m^2)]. Overweight was defined as a BMI > 25 kg/m^2 (Hwang *et al*., 2006). Hypertension defined as mean systolic blood pressure ⩾140 mm Hg or mean diastolic blood pressure ⩾90 mm Hg. Hyperlipidemia was defined as patients having cholesterol level ≥ 200 mg/dl. According to family income (monthly) patients were divided into three

categories as low income (≤ 10,000 PKRs), middle income (10,000-20,000 PKRs) and high income group (≥ 20,000 PKRs). Fasted blood samples (3ml-5ml) of all participants were collected by special staff and were sent to concerning laboratories of hospitals for lipid profile and blood sugar measurement. Those subjects having fasting glucose ≥ 126 mg/dl were considered hyperglycemics and those having history of diabetes mellitus as well as using glucose lowering medicines considered diabetics. Diabetes defined as patients taking antihypertensive drugs or having fasting blood sugar ⩾7.0 mM. Disease treatment cost was expressed in Pakistani Rupees (PKRs) and for international level it was converted to US dollar at a rate of 98.50 PKRs = 1US$.

3.2 Second phase (Genetic analysis)

For second phase (genetic analysis) of the study, total of 384 Pakistani subjects (Central Punjabi population) consisted of each 192 MI patients and healthy controls were recruited and analyzed. Blood sampling was carried out from Punjab Institute of Cardiology and Jinnah Hospital Lahore Pakistan. The diagnostic criteria for patients were same as described above. The complete clinical and demographic history of all subjects was recorded. Control subjects were individuals without a history of CHD and were in good health. They were frequency-matched to patients by sex and age (in 5-year bands). This study was conducted as per Good Clinical Practice (GCP) guidelines (Declaration of Helsinki). Patients not willing to sign consent form and with previous illness associated with hepatic and renal failure, HIV, cancer were excluded from the study.

3.3 Blood Sampling

Blood was drawn after an overnight fast but within 48 hours of admission to the hospitals and before coronary angiography for genetic analysis. In second phase of study about 3 to 5 ml of blood was collected in EDTA coated tubes and was stored at 4.0^{o}C for further process of DNA extraction. Fasting concentrations of total cholesterol, plasma triglyceride, and blood sugar were measured for cases and control at the laboratory of Punjab Institute of Cardiology Pakistan.

3.4 DNA extraction

DNA was isolated using modified extraction procedure (Sambrook *et al*., 1989). In the first step 500 µl of blood sample was mixed with 500 µl of TE buffer and incubated at room temperature for 15 min, centrifuge the sample at 25^{o}C with 13500 rpm for 5-10 min. Then pellet was broken after discarding supernatants. Pellet was dissolved in 375 µl of 3M sodium acetate. Then 25 µl of 10% SDS and 5-10 µl of proteinase K (10 µg/µl) were added and incubated at 37^{o}C overnight in a shaking water bath. Chilled chloroform: isoamyl alcohol (24:1) was added. Mixed and centrifuged at 25^{o}C with 13500 rpm for 5-10 min. Three layers were visualized after centrifugation. The upper layer contained DNA. The DNA layer was carefully transferred into a new labeled eppendorf. Chilled absolute ethanol was added by gently inverting eppendorfs. The dried DNA pellet was dissolved into 50-100 µl low TE buffer, incubated in a shaking water bath at 70^{o}C for 30 min. Detailed procedure is described in Annexures II. Concentration of DNA was measured by nanodrop method.

3.5 Selection of Genes and SNPs

Selection of SNPs was based on the reported genetics association with MI from HapMap data bank and National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). Those SNPs were selected from the genes which were reported to be involved in regulating plasma triglycerides, HDL-C, LDL-C or playing role in the enhancement of MI risk factors for example obesity, depression and hypertension. The list of 22 SNPs in 9 candidate genes chosen for determination of the genetic association between cases and control has been shown in Table 3.1.

3.6 Oligonucleotide primers

Primers for 1st and 2nd poymerase chain reaction (PCR) were designed using primer 3.input 0.40 (http//frodo.wi.mit.edu/primer 3) and supplied by Bio Basic, Canada (Table 3.2). The specificity of the primers were further examined and confirmed through sequence alignment of them with the human genomic reference sequence using BLASTN program and the fidelity of PCR amplicons was verified by agarose gel electrophoresis.

Table 3.1 Selected SNPs for myocardial infarction genetic association study

SNP NO	SNP ID	chr	Position	Gene
S1	rs4977574	9p21.3	22188574	*CDKN2A/B*
S2	rs2891168	9p21.3	22188619	*CDKN2A/B*
S3	rs2383206	9p21.3	22115026	*CDKN2A/B*
S4	rs2383207	9p21.3	22115959	*CDKN2A/B*
S5	rs10811656	9p21.3	22124472	*CDKN2A/B*
S6	rs10757278	9p21.3	22124477	*CDKN2A/B*
S7	rs1333049	9p21.3	22125503	*CDKN2A/B*
S8	rs10757283	9p21.3	22134172	*CDKN2A/B*
S9	rs1333047	9p21.3	22124504	*CDKN2A/B*
S10	rs10757277	9p21.3	22124450	*CDKN2A/B*
S11	rs10757279	9p21.3	22124630	*CDKN2A/B*
S12	rs3135506	11	116662407	*APOA5*
S13	rs1558861	11	116607437	*APOA5*
S14	rs662799	11	116663707	*APOA5*
S15	rs10750097	11	116664040	*APOA5*
S16	rs599839	1P13.3	109818530	*PSRC1*
S17	rs646776	1P13	109822166	*CELSR2*
S18	rs3846663	5q13.3	7655726	*HMGCR*
S19	rs2271293	16	67902070	*NUTF2*
S20	rs6511720	19	11202306	*LDLR*
S21	rs1537375	9	22116071	*ANRIL*
S22	rs4986790	9	120475302	*TLR4*

Chr =chromosome, SNP = single nucleotide polymorphism

Table 3.2 Primers used for sequencing and OLE

SNPs	1st PCR Primers Sequence (5'- 3')	Product size	Nested PCR Primers Sequence (5'- 3')	Product size	Annealing Tm (◦C)
S1	F: ggcccacatcacctccttat	1696	F: ttgcttgctggttcccagag	797	56
	R: tagacgtgcccagctattgg		R: tgaatggcatgttttccagcg		
S2	F: aagcaggtagcaggtagtgc	1705	F: ccatgctttctgaaacaacacg	795	58
	R: ccagctccccgtcaattcat		R: cgtgcccagctattggctat		
S3	F:ctgcttgcctcacaggatct	1718	F:gcaagccacatgcccctaag	400	60
	R:gtttatgcatgcgtcctggc		R:ttaagggacaggaagaatcaggac		
S4	F: acacttatgccaggacgcat	1321	F:cagcagggaagtgattccga	741	56
	R: tgggccaagttgtctcaagt		R:ggggagtacagactaccttgtg		
S5	F: aggtgctgctattaggatggc	1216	F: tgaggtcgcaactaaaagcca	559	56
	R: ggcaggccacacttgtgtaa		R: tccacgctgttcccaagtag		
S6	F: aggtgctgctattaggatggc	1216	F: tgaggtcgcaactaaaagcca	539	58
	R: ggcaggccacacttgtgtaa		R: tccacgctgttcccaagtag		
S7	F: cctggctacttgggaacagc	1793	F: gaccttcatgctattttgaggag	591	58
	R: tgctctcaactaaaccttggc		R: acaatatgtctggcagatcttgga		
S8	F: tctgagagccacagttgtcc	1774	F: aaccttcagccacctctctg	779	56
	R: tcctgattaaccccaaccagc		R: tctctttctgtgtgggatgc		
S9	F: aggtgctgctattaggatggc	1710	F: tgaggtcgcaactaaaagcca	760	56
	R: ggcaggccacacttgtgtaa		R: tccacgctgttcccaagtag		

Table 3.2 continue

SNPs NO	1st PCR Primers Sequence (5'- 3')	Product size	Nested PCR Primers Sequence (5'- 3')	Product size	Annealing Tm (◦C)
S10	F: aggtgctgctattaggatggc	1710	F: tgaggtcgcaactaaaagcca	760	60
	R: ggcaggccacacttgtgtaa		R: tccacgctgttcccaagtag		
S11	F: aggtgctgctattaggatggc	1710	F: tgaggtcgcaactaaaagcca	760	56
	R: ggcaggccacacttgtgtaa		R: tccacgctgttcccaagtag		
S12	F: ggtcttgctcaaggctgtct	1128	F:gggcgctaaagagcccaggat	650	58
	R:gaactgttcctggggtctgg		R:tagcctccttgactcacctaggtca		
S13	F: tgggaatccgtggtgtgatg	1080	F: ctctctctccttcacaaggaact	601	58
	R: cagaaccaagcgtgacaacc		R: ggaggaaaaggatgcaggacc		
S14	F: tgcttcccaggagctttacg	1769	F: agcttcactacaggttccgc	529	60
	R: ggacaggccacttgatctcc		R: taaaggctcagggaatgccg		
S15	F:gggcccctctcttacacaac	1376	F:ccttcttcggccttcacctt	775	58
	R:aaggtgaaggccgaagaagg		R:gcccgagctggcctacattt		
S16	F: gggaggccctacaccaaatc	1392	F: gaccaacctgaccaacatgg	539	60
	R: ccagggacaggaggaagaga		R:tggacatttccacgtgagcg		
S17	F: tccaaccagcctctcaatgc	1216	F: gggcaaagagaagtgggact	539	56
	R: ccaggtgtttgctcagttgc		R: aggtggctgaacaagaaggg		

Table 3.2 continue

SNPs NO	1st PCR Primers Sequence (5'- 3')	Product size	Nested PCR Primers Sequence (5'- 3')	Product size	Annealing Tm (◦C)
S18	F: atatcagctgcaccatgcca	1347	F: tcaggcatagagtccacaagc	698	56
	R: gtcgggctattcaggctgtc		R: tgccaatgctgccataagtg		
S19	F: gttctgcccttgaaggctgt	1769	F: tgaccccatgggtatccaga	529	56
	R: cagaaggggtgggaaaggag		R: ccttcccacgtaaggcatga		
S20	F: tcacaaaggcgacgacaagt	1707	F: caaggggttggctttgact	669	60
	R: gctgctggtcggttacatct		R:tctaagtgaaggcttgcggt		
S21	F: caaggagggaagactgggga	1483	F: cagcagggaagtgattccga	790	58
	R: aggacctttatggcctcagc		R: tccgtttgatatacaccgtttgag		
S22	F: tattgcacagacttgcgggt	1348	F: tccatcgtttggttctgggag	690	58
	R: tgaggaccgacacaccaatg		R:cctggaaagaattgccagcc		

OLE=one base label extension, F = forward primer, R = Reverse primer, Tm = temperature, bp = base pair

3.7 Genotyping by OLE and verified by sequencing

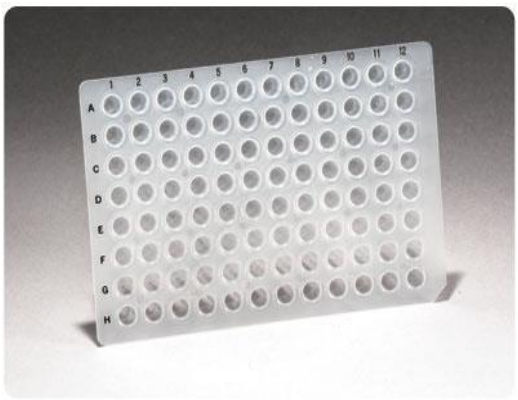
All genotyping {Sequencing and One Label Extension (OLE)} was performed at Applied Genomic Center of Hong Kong University of Science and Technology (HKUST) Hong Kong. Following the procedure described below.

3.7.1 Master Mix (5x) composition for PCR

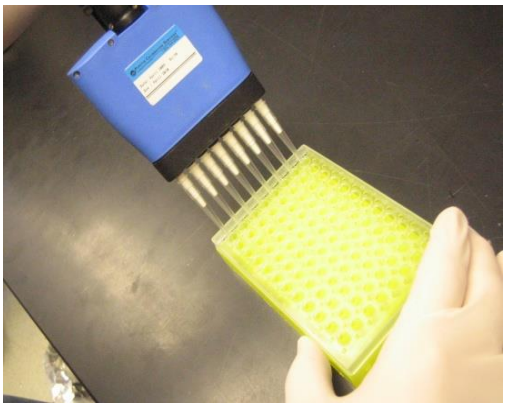
- H_2O 360 µl
- dNTPs (10mM) 100 µl
- $MgCl_2$(0.2M) 40 µl
- 10x buffer 500 µl

3.7.2 1st PCR

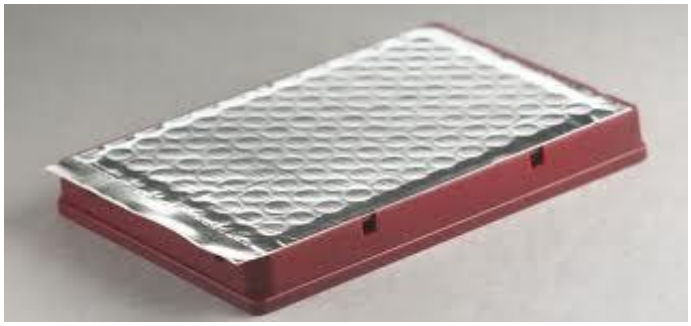
A 96 well plate was used for optimization process (Annexures III). After optimization DNA fragments for each primer were amplified through 1st PCR. Amplification was carried out in a 20 µl PCR mixture containing 50-60 ng of genomic DNA, 75 nM of each primer (PCR primers are listed in Table 3.2), 50 nM of each deoxyribonucleotide triphosphate (dNTPs: Promega USA U1515), 2.5 mM magnesium chloride ($MgCl_2$, Sigma: M2670) and 1U Taq DNA polymerase (Amersham Bioscience, Uppsala, Sweden). The program of PCR consisted of one cycle of initial denaturation at 95°C for 5 min, followed by 30 cycles of 30 seconds at 95°C, 30 seconds at 56°C (gradient PCR), 2 min at 72°C and final extension step at 72°C for 8 min (Applied Biosystem thermal cycler model# 9902, Singapore). As shown in Figure 3.1.



(a)



(b)



(c)

Figure 3.1: (a) 96-well PCR plate **(b)** samples loading **(c)** seal plate with foil seal after loading reaction mixture

3.7.3 Purification of 1st PCR product

Absolute ethanol was used for the purification of PCR products and the mixture was kept at -20°C for at least 1 hour. Take out PCR product and after centrifugation at 3500 rpm (eppendorf 5810 centrifuge, Germany) for 30 min at room temperature, the precipitates were washed out two times with 70% ethanol and centrifugation was done between washes at 3500 rpm for 20 min (Figure 3.2). Vacuum was applied for at least 20 min until wells were completely dry. 20 µl H_2O (Millipore USA) was added to elute the purified PCR product. Heat the product in PCR machine by run the program 5510 (5m at 55°C) and was stored at -20°C until use.

Figure 3.2: Centrifuge machine used for short spin and for purification of PCR product

3.7.4 Nested PCR (2nd PCR)

DNA fragment was amplified through nested PCR using genomic DNA as template in the first round of amplification (Lo *et al*., 2007) and using amplicons from the first round as the templates in the second round of amplification.

3.7.4.1 Nested PCR (Ingredients of reaction mixture)

PCR Components	volume of each well		volume of half plate
• Forward primer			3.25 µl (Each primer)
• Reverse primer			3.25 µl (Each primer)
• Master mix	4 µl	50x	200 µl
• H_2O	14.5µl	_____	725 µl
• Taq pol	0.3 µl		15 µl
• DNA	0.6 µl		
	20 µl		1000µl

Amplification of DNA fragment was carried out in a 20 µl PCR mixture containing 50-60 ng of genomic DNA, 125 nM of each primer , 200µM of each dNTPs (Promega USA U1515), 2.5 mM $MgCl_2$ (Sigma: M2670), 10mM Tris HCl, 50mM, Potassium Chloride (KCl) and 1U *Taq* DNA polymerase (Amersham Bioscience, Uppsala, Sweden). PCR amplification consisted of denaturation at 95°C for 5 min, followed by 30 cycles of 30 sec at 95°C, 30 sec at 56°C, 1 min at 72°C and finally at 72°C for 8 min. The annealing temperature was modified according to different SNPs for 2nd PCR, the protocol remained same except in the final step one min instead of 2 min at 72°C. The ethanol purification was carried out (as described above) and amplicon was stored at 4°C till further use (DNA sequencing and OLE assay).

3.7.5 Agarose Gel Electrophoresis

It was used to determined the PCR products (presence or absence) and quantify the size (length of the DNA molecule) of the product (Annexure IV).

3.7.6 Direct DNA sequencing

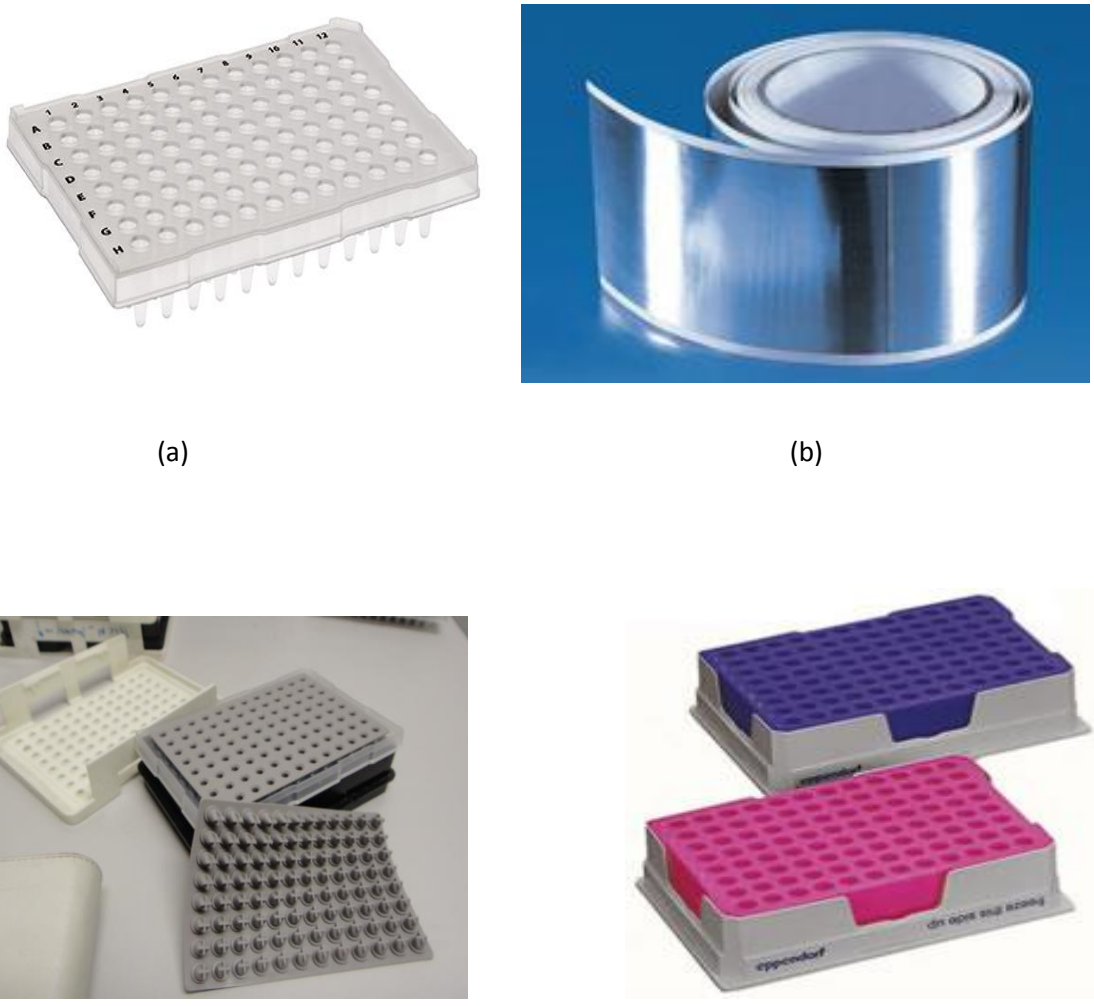
SNP genotyping was executed by direct sequencing of nested PCR Product (Figure 3.3). Each sequencing reaction contained 0.75 µl of Big Dye (Applied Biosystem UK 1201096), H_2O (Millipore, USA) 14.5 µl, sequencing buffer 3.00 µl (Applied Biosystem UK 1203142), 240nM forward/reverse primer (refer to Table 3.2 for sequencing primer sequence) and 1.5 µl purified PCR product. Each cycle of sequencing reaction consisted of initial denaturation at 96 °C for 1 min, followed by 25 cycles each of 10 sec at 96 °C, 5 sec at 50 °C and 4 minutes at 60 °C. After sequencing reaction plates were taken out and short spinned.

Figure 3.3: Genetic analyzer

3.7.6.1 Purification for sequence reaction and sequencing

Ethanol precipitation was carried out to clean-up the post-sequencing mixture. After sequence reaction 45µl of 100% ethanol was added to each well and placed at -20◦C for 1 hour to precipitate the DNA. The plate of sequence reaction was taken out and placed on bench for one minute. The centrifugation was done at 3500 rpm for 30 minutes at room temperature to get the precipitates of DNA. Precipitates were washed three times

with 75% ethanol at 3500 rpm for 20 min. The plate was rapped with a piece of paper, spin down all solution for 7 sec. Each plate was dried for 20 min in a Vacuum pump. Each air-dried sequencing sample was dissolved in 10 µl Hi-Deionized Formamide [Hi-Di (Applied Biosystems Inc)]. After adding Hi-Di the plate was sealed and denatured at 95°C for 5 min in 96-well thermo cycler then take out and quickly transferred to coolers which were stored at -20°C (Figure 3.4). At least for 3-5 min the plate was placed in cooler and settled in the rack, adjusted in genetic analyzer to sequence the fragment of interest (Model 3130 Genetic Analyzer (Applied Biosystems UK).



(a) (b)

Figure.3.4: (a) Sequence plate (b) foil film (c) racks for sequencing (d) small coolers (ice racks).

3.8 Second method for SNP genotyping

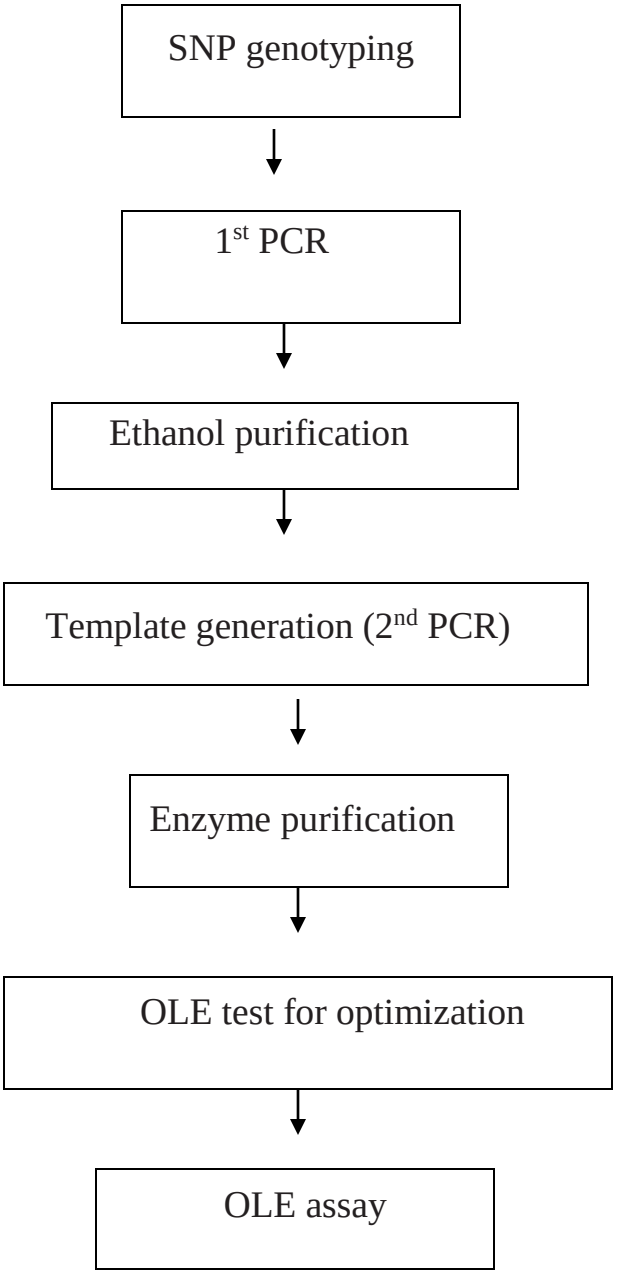


Figure 3.5: Sketch for SNP genotyping by OLE assay

3.8.1 Primers for OLE assay

Primers (forward and reverse) for OLE assay were designed in which 3' end of the each primer was directly adjacent to the nucleotide bases to be identified (Table 3.3).

Table 3.3 Oligonucleotide primers for one label extension method

SNP no	Forward primers	Primer length (bp)	Reverse primers	Primer length (bp)
S1	acatcaaatgcattctatagc	21	agagtgactggaacatcctg	20
S2	acaaaagatgtcctgtttggaac	23	tgaagtaactgatacagagtt	21
S3	ttccttagaaatgttattgtagt	23	caggattcaggccatcttgcaaa	23
S4	tactcctgttcggatcccttc	21	tgaagtaactgatacagagttgg	23
S5	caagtcagggtgtggtcattc	21	ttctgcatcgctgcttacc	19
S6	cagggtgtggtcattccggta	21	gtcttgattctgcatcgctgc	21
S7	aaccatatgatcaacagtt	19	tctgcgagtggctgctttt	19
S8	ggatggggaaggtttttgacttta	24	cccaaaatttgttctgagaatatc	24
S9	atgcagaatcaagacagagt	20	gagagagaaagaaac	15

Table 3.3 continue

S10	Aggccagacagggctgtg	18	gaccacaccctgacttg	17
S11	tctatttttttaaaaaact	18	aaaacaagagaaaagt	16
S12	cctttccgtgcctgggtggcc	21	ccctctccacagcgtttt	18
S13	atcagcacactttgaacatta	21	ccagttggagtgagctgggcc	21
S14	cccaggaactggagcgaaagt	21	ttttcctcatggggcaaatct	21
S15	atctgctgccacataaaaccac	22	tataaaggctcagggaatgc	20
S16	gaaataggagcaggatc	17	attctctgtatatctggaagt	21
S17	tgggagcagtgtcatggacat	21	aagcctgtccctctgcc	17
S18	gttctattctgatgccatta	20	tcaactaaaaacagggcaact	21
S19	gggcccagggtcaatggggta	21	acattacactatcaacatctt	21
S20	caatcaacctcttccttaag	20	taagacttccttaacatttt	20
S21	tcaacattctcttagcttctt	21	ccaattttggaaagagat	18
S22	acttagactactacctcgatg	21	acaattaaataagtcaataata	22

bp; base pair

3.8.2 PCR reaction for OLE template generation

After 1st PCR and ethanol purification (same protocol mentioned above) next step was template generation which was same as nested PCR with minor change in concentrations. Following reaction mixture was used:

3.8.2.1 Template generation (2nd PCR), Reaction mixture

• Forward primer	0.75 µl
• Reverse Primer	0.75 µl
• Master mix	10.0 µl
• H_2O (Millipore)	36.25 µl
• Taq polymerase	0.75 µl
• DNA template	1.5 µl
Total volume	50 µl

Above reaction mixture was used for one reaction (one genotype or one base pair possibility) in one PCR tube. For each SNP we make three PCR tubes according to three genotypes (base pair possibilities of sequence result) and used three DNA templates from purified 1st PCR product then labeled them as homozygous dominant, homozygous recessive and heterozygous for each SNP. Run PCR program as mentioned above for nested PCR.

3.8.3 Enzyme purification

PCR products were treated with Exo nuclease I (ExoI) and Shrimp Alkaline Phosphtase (SAP). After template generation (2nd PCR) enzyme purification was done. Following reaction mixture was used for enzyme purification.

Mixture I

- SAP buffer (10X) 10 µl
- H_2O (Millipore USA) 38 µl

Mixture II

- EXO 1(5U/ µl) 0.3 µl
- SAP (Shrimp Alkaline Phosphatase: 1U/µl) 2.25 µl

Total volume	50.55 µl

The reaction mixture was transferred to each nested PCR tube. Now total volume of each tube was 100 µl. The above mixture was vortex and short spin for several sec. Run following program named as 3785 in PCR machine (Applied Biosystem thermal cycler model# 9902, Singapore) consisted of initial temperature at 37°C for 45 min followed by one cycle of 15 min at 85°C.

3.8.4 OLE test

After enzyme purification, for every SNP appropriate OLE test (optimization) was performed in PCR tubes to choose best method for OLE assay i.e., following procedure was used for genetic variant rs6511720 (Table 3.4).

Reaction mixture for OLE test

- Therminator buffer (TE buffer) (10X) 2.0 µl
- Taq pol 0.3 µl
- R110-labeled dideoxy-terminators (10 µM) 1.0 µl
- Primer F/P (5 µM) 1.0 µl
- H_2O (Millipore USA) up to 20 µl
- DNA template 1.5 µl

Total volume	20 µl

3.8.4.1 SNP Rs6511720 (NCBI sequence)

ATCAACCTCTTCCTTAAGA[G/T]AAAATGTTAAGGAAGTC

For this genetic variant (rs6511720) following four methods were used for OLE assay (according to presence of bases G or T for current sequence) (Table 3.4).

Table 3.4: Different methods used for OLE assay

Methods	Labeled nucleotides
OLE one base F	dGTP*, d UTP*
OLE one base R	dCTP*, d ATP*
OLE two base F	dGTP +, d ATP*/ dTTP, d ATP*
OLE two base R	dCTP + d UTP*/ dATP, dUTP*

Here *= represent florescent dye, F = forward primer, R = reverse primer

All work was run in dark. After vortex and short spin all these PCR tubes were transferred in to PCR machine and run OLE reaction by use of the following program:

- Denaturation 95°C 5min
- Denaturation 95°C 30 sec } 30 cycles
- Elongation 55°C 30 sec

The above reaction mixture was prepared in appropriate PCR tube. We used four PCR tubes for each OLE reaction was done according to schematic diagram 3.6.

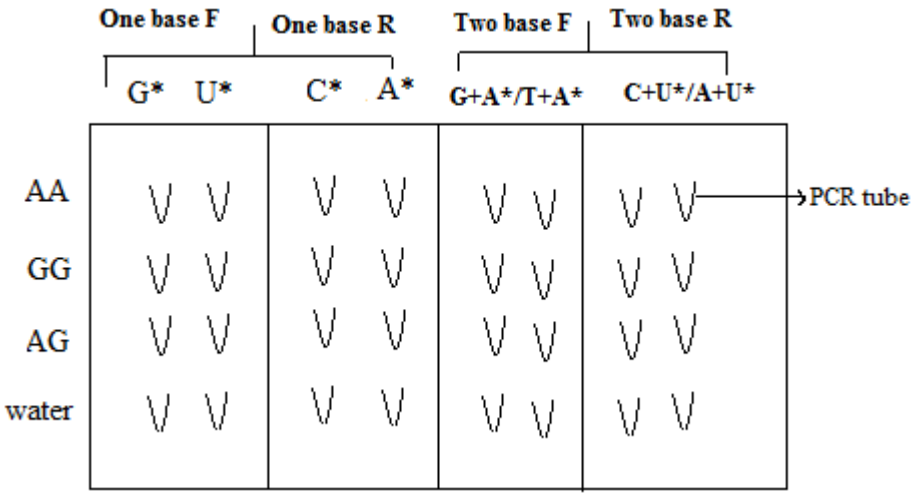


Figure 3.6: Sketch of PCR tubes with reaction mixtures for OLE test, V = PCR tube, * = dye, F = forward primer, R = reverse primer, (AA, GG, AG) = template DNA with known genotypes

After OLE reaction the PCR tubes were taken out and 10µl product from PCR tubes was transferred into each well of black OLE plate of 384 wells (Figure 3.7). The plate was sealed and short spin. Florescence polarization (FP) was measured by using Wallac VICTOR^{2}V 1420 Multi label Counter (PerkinElmer, Boston, USA) (Figure 3.8) (Annexure V).

Figure 3.7: OLE plate

Figure 3.8: Multilabel counter

3.8.5 OLE assay for SNP genotyping

OLE reaction (Yu *et al*., 2006) was carried out in 384-well plates in a 20 µL reaction mixture containing (a) 60 ng PCR-amplified DNA template, (b) 0.3 U Taq polymerase (TaKaRa Dalia, China), (c) 250 nM OLE primers (5µM) (OLE primers are listed in Table 3.3), (d) 4 µL 5× OLE reagents (Pharmaco Genetics Ltd., Hong Kong) according to manufacturer's protocol.

(e) 50nM TAMARA-dNTP (N for A/C/G/U), full names are following

- Aminoaallyl-dUTP-5/6-TAMARA(TAMARA-dUTP)
- 7-propargylamino-7-deza-dATP-5/6-TAMARA (TAMARA-dATP)
- 5-Propargylamino-dCTP-5/6-TAMARA (TAMARA-dCTP)
- 7-propargylamino-7-deaza-dGTP-5/6-TAMARA(TAMARA-dGTP)
  Jena Biosciences,Germany.

After initial denaturation at 95°C for 3 min, primer extension was carried out by 30 thermal cycles consisting each of 95°C for 30 sec and 55°C for 30 sec. The FP value of the OLE reaction was read from the 384-well plate by using the same Wallac VICTOR^{2}V as described above (PerkinElmer, Boston, USA) without any purification (Annexure VI). OLE assay is summarized in Figure 3.9.

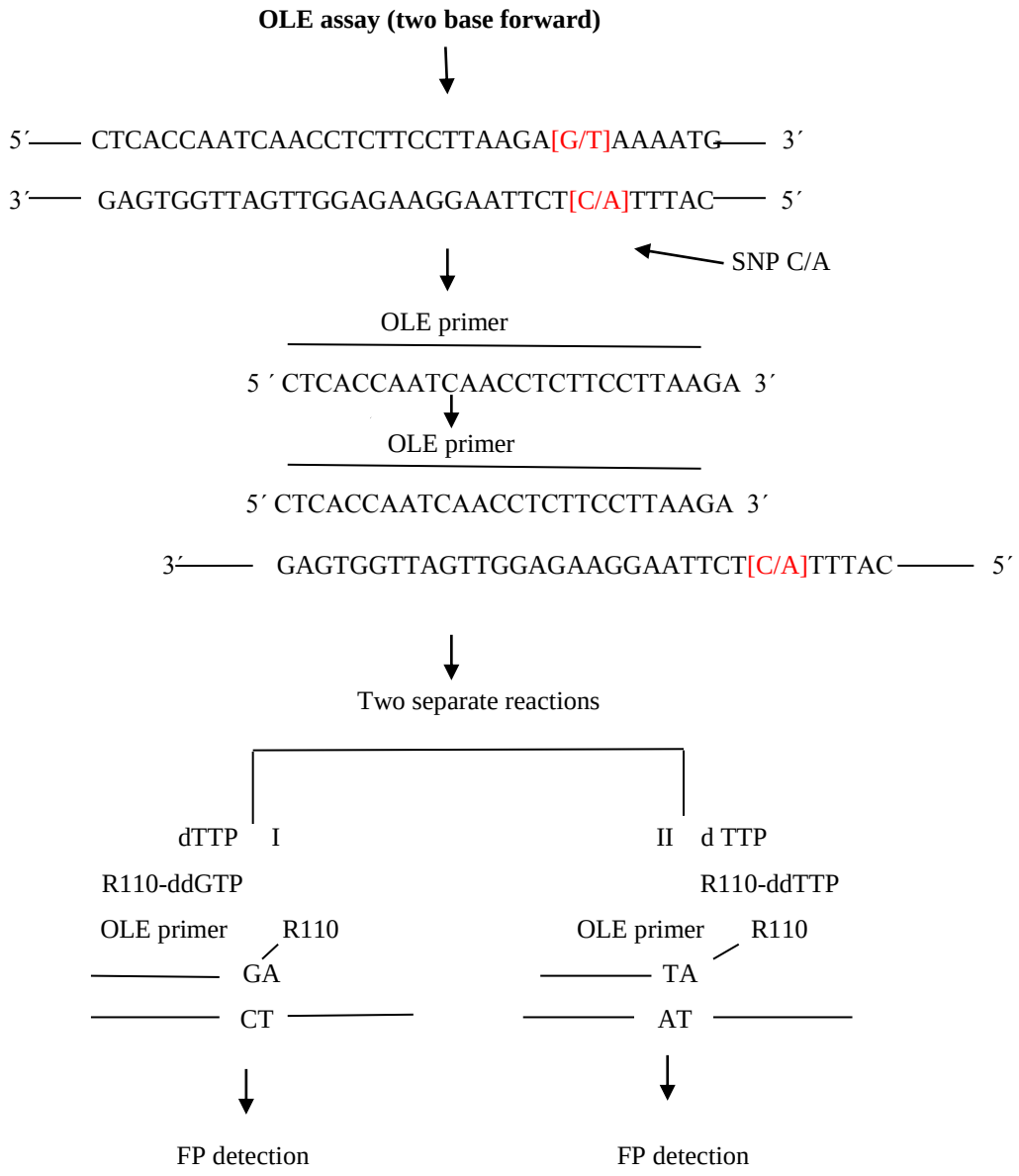


Figure 3.9: Schematic presentation of OLE two base extensions

3.9 Statistical analysis

Demographic data (Age, gender, marital status, BMI, education, income, cost, family history and smoking) was calculated by the use of R version 2.11 software for all patients enrolled in the study. Wilcoxon signed-rank test for continues values (BMI, age) and Chi-square test for discrete values (gender, marital status, family history, smoking, history of diabetes, hypertension and hyperlipidemia) was used. Continuous values were presented as mean ± standard deviation (SD) and categorical values were exhibited in numbers as well as percentages. Association studies for the relationship between one continuous variable (BMI, cost) and other independent variables (family history, rural and urban population and income) were carried out by 'Kruskal Wallis' and 'Mann Whitney U' test. The box and whisker plots were created for association between different income groups of patients and for comparison of BMI with family history and urban and rural population respectively by using R version 2.11. The *P* value less than 0.05 was considered as statistically significant.

3.9.1 Hardy-Weinberg Equilibrium

Exact test of Hardy-Weinberg Equilibrium (HWE) was performed by the program GENEPOP version 3.4 (Raymond and Rousset, 1995) for control samples, with the following parameters: dememorization number (1000), number of batches (100) and number of iterations per batch (1000). The Hardy–Weinberg principle states that in a population both allele and genotype frequencies remain constant generation after generation with the absence of external disturbing influences. For a single locus with two alleles: the frequency of major allele (A) and minor allele (a) are denoted as p and q; frequency (A) = p; frequency (a) = q; $p + q = 1$. If the population is in equilibrium, we will have frequency (AA) = p^2 for the AA homozygote in the population, frequency (aa) = q^2 for the aa homozygote, frequency (Aa) = $2pq$ for the heterozygote, and $p^2 + 2pq + q^2 = 1$. If the observed allele and genotype frequencies in a population are significantly deviated from this equilibrium, probably non-random mating, mutations and selection are present in the population or the sample size of the population is too small. For this reason, all the control groups for the disease association analysis should pass this HWE test.

3.9.2 Genetic association

The disease association analysis was carried out by using software package UNPHASED version 2.404. Additional soft ware SHEsis, Chi sq 2/2 were employed in order to compare allele and genotype frequency of cases and control for all SNPs. if any allele (or genotype) have higher frequency in cases than control; then we called it is associated in causing disease. Permutation procedures of 100 replicates were employed to obtain empirical *P* values by means of UNPHASED program**.** To evaluate the association between *APOA5* and plasma triglyceride levels, a multiple logistic regression model was fitted. Demographic parameters were analyzed for second phase of study (comparison between cases and control) by using SPSS v 18.0 package.

3.9.3 Linkage disequilibrium

Two common parameters of LD: D′ and r^2 were calculated by SHEsis for SNPs in the same gene. D′ is scaled from 0 to 1 by the observed marker allele frequencies such that a D′ of zero indicates no LD and a D′ of 1 indicates the maximum possible LD given those allele frequencies. Another measure, r^2 is a squared correlation coefficient and denotes the ability of alleles at one locus to predict alleles at the second locus on similar choromosomes.

CHAPTER 4

RESULTS

4.1 Demographic distribution of MI (First phase)

The data collected from North of Punjab Pakistan indicated out of 515 patients from rural and urban areas, 356 (69.13%) were males as compared to females 159 (30.87%) in this phase of study. The mean age of participants of rural areas was 56.8 years and of urban areas was 55.7 years with a range of 20-80 years. The MI was significantly higher among males as compared to females in both urban and rural population (P = 0.015) (Figure 4.1). According to age MI patients were divided in to three groups. Groups I consisted of 20- 40 years of age and contain 12.23% of MI patients. Groups II comprises 41-60 years of age have 53.79% patients and group III have patients ≥ 61 years with 33.98% patients. In case of BMI, obesity (BMI > 25) was found among 43.11 % participants with MI. In addition 72.04 % patients were found with previous family history of heart attack. There was a low literacy rate among MI patients. The 39.03% patients were found illiterate and 41.36% patients were without any job (Table 4.1).

4.1.1 CO-morbid conditions and family history related to MI

The patients with ST Segment Elevation Myocardial Infarction (STEMI) were 56% more as compared to Non Segment Elevation Myocardial Infarction (NSTEMI) 44% (P > 0.05). Smoking was more common (32.8%) in MI patients of rural areas as compared to urban areas (28.1%). However, there was no significant difference of smoking (P = 0.20) between rural and urban areas. Overall 60.9% patients were smokers. The 70% patients were having sedentary life. There was high prevalence of hypertension (37%) as compared to diabetes (19.4%) in MI patients of Pakistani population (Table 4.2). The 26% of selected MI cases had hyperlipidemia (cholesterol level ≥ 200 mg/dl) while 17% patients had two or more risk factors (Table 4.2).

Table 4.1 Demographic distribution of myocardial infarction

Parameters	**Rural (%)**	**Urban (%)**	**Total (%)**
Subjects	265 (51.46)	250 (48.54)	515(100)
Gender			
Male	170 (33.0)	186 (36.1)	**356 (69.13)***
Female	95 (18.4)	64 (12.4)	159 (30.87)
Age			
20-40	38 (7.38)	25 (4.85)	63 (12.23)
41-60	136 (26.41)	141 (27.38)	**277 (53.79)**
≥ 61	91 (17.67**)**	84 (16.31)	175 (33.98)
BMI			
≤ 25	205 (39.81)	88 (17.09)	**293 (56.89)***
>25	60 (11.65)	162 (31.46)	222 (43.11)
Family History			
+ve family history	235 (45.63)	136 (26.41)	**371 (72.04)***
-ve family history	30 (5.83)	114 (22.14)	144 (27.96)
Education			
Illiterate	99 (19.22)	102 (19.8)	**201 (39.03)**
Primary	77 14.95)	59 (11.46)	136 (26.41)
College & above	89 (17.28)	89 (17.28)	178 (34.56)
Marital. Status			
Married	251 (48.74)	240 (40.60)	**491 (95.34)***
Unmarried	14 (2.72)	10 (1.94)	24 (4.66)
Employment status			
Jobless	144 (27.96)	69 (13.40)	**213 (41.36)**
Laborer	8 (1.55)	76 (14.76)	84 (16.31)
Housewifes	12 (2.33)	27 (5.24)	39 (7.57)
Office job/business	101 (19.61)	78 15.14)	179 (34.76)

Results are presented in numbers of patients (percentage). **Rural** = Rural population (Faisalabad, Gujranwala, Gujrat and Sialkot), **Urban** = Urban population (Lahore, Islamabad), **BMI** (Body mass index) was considered as being normal at ≤ 25 and overweight at > 25 for both men and women according to WHO: Bold letters are representing the higher percentage among groups. (* = $P < 0.05$)

Table 4.2 Co-morbid conditions and types of myocardial infarction

Parameters	**Rural (%)**	**Urban (%)**	**Total (%)**	**Chi-sq *P* values**
No. of patients	265(51.4)	250(48.5)	515 (100)	
Types				
STEMI	152(30)	138(27)	290(56)	0.65
NSTEMI	113(22)	112(22)	225 (44)	
Smoking				
Smokers	169(32.8)	145(28.1)	314 (60.9)	0.20
Non smokers	96(18.6)	105(20.3)	201 (39)	
Life style				
Sedentary	151(29)	213(41)	364(70)	0.00*
Physical activity	114(22)	37(7)	151 (29)	
Other Complications				
Hypertension	90(17)	100(19.4)	190 (37)	0.00*
Diabetes	35(7)	65(13)	100(19.4)	
Hyperlipidemia	100(19.4)	35(7)	135 (26)	
Risk Factors≥ 2	40(8)	50(8)	90 (17)	

Results are presented in numbers of patients (percentage) * = $P < 0.05$

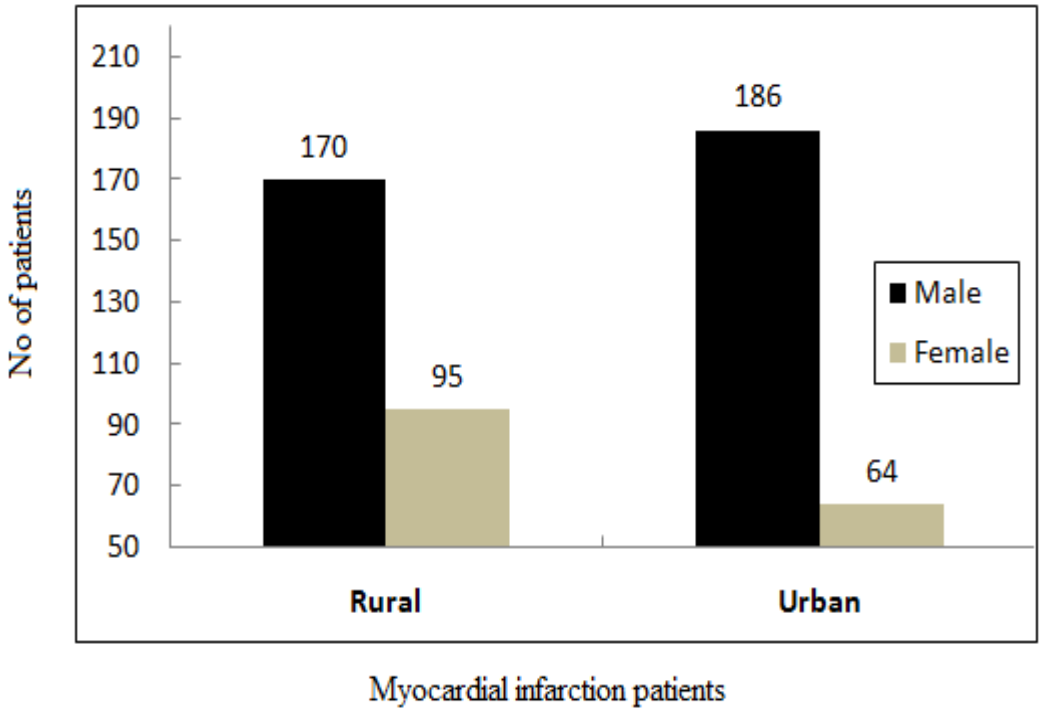


Figure 4.1: Prevalence of myocardial infarction in Urban and rural areas of Pakistan**.**

The BMI and family history of MI patients was compared. In comparison of this it was found participants with 1st degree relative positive family history experienced MI even at BMI $\leq$ 25 kg/m^2 (Figure 4.2). In the next step BMI was compared among urban and rural MI patients, it was noticed patients of urban population were significantly more overweight as compared to rural patients ($P < 0.05$) (Figure 4.3).

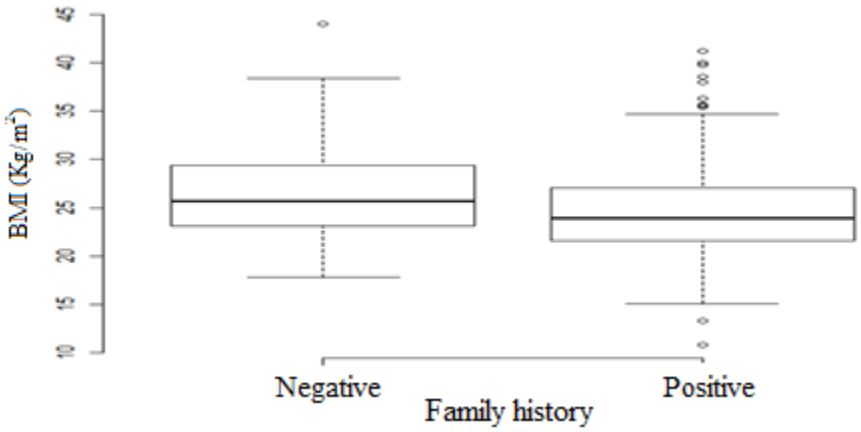


Figure 4.2: Box and whisker plots for Comparison of BMI (Kg/m^2) with family history. The boxes represent a 25–75% range of the values and the whiskers represent the minimum and maximum values. The median values are marked with a dark line in the boxes. Excluding outliers are shown with circles.

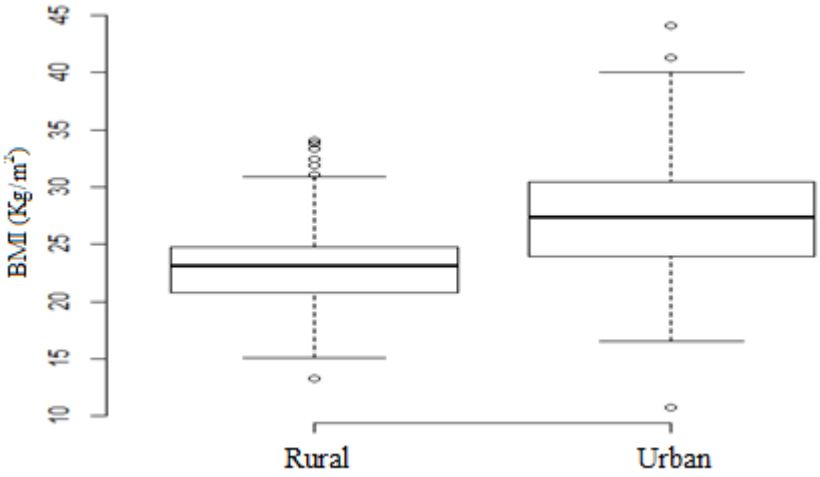


Figure 4.3: Box and whisker plots for comparison of BMI (Kg/m^2) with rural and urban population (P = 0.0041). Key as for Figure 4.2

4.1.2 Economic burden

Relationship between monthly income and overall cost (ECG, CT scan, Angiography, surgery and medication) of MI patients was presented in Figure 4.4. All income classes (lower, middle and higher) were spending almost equal amount for the treatment of disease. The mean cost of monthly medicine and physicians after three months check-up was 2381.132 PKRs (24.24 USD) with annual cost was 9524.53 PKRs (96.96 USD).The mean cost for heart surgery was 600 thousand (6108.19 US$).

Figure 4.4: Box and whisker plots for comparison between surgery cost and monthly income of MI patients (K= 1000 PKRs). Key as for Figure 4.2 {mean cost for surgery = 600 thousand (6108.19 US$)

4.2 Second phase (Genetics study)

In the second phase, total of 384 subjects (192 each of MI and control) were genotyped (sequencing and OLE assay) for all 22 SNPs selected for current study. Out of 22 SNPs only 3 did not show two base pair possibilities (homozygous dominant and recessive) in selected population. Therefore, these three SNPs rs6511720 (S20), rs1537375 (S21) and rs4986790 (S22) were excluded from any disease association analysis. Selected individuals were in the age group from 20-80 years with mean age of cases and control was 55.6 and 53.4 years respectively.

4.3 Clinical characteristics

The clinical characteristics for second phase of study among cases and control subjects are listed in Table 4.3. The cases had significantly higher ($P < 0.05$) cholesterol, LDL-C, triglycerides, BMI, smoking, hypertension and diabetes as compared to control. Detail of optimized primers for annealing temperature and sequenced primers used are summarized in Table 4.4.

Table 4.3 Basic characteristics of MI and normal controls in Pakistani population

Variables	**Case (n=192)**	**Control (n=192)**	**Total(n=384)**
Age (year)	55.6±0.8	53.44±0.8	54.45±0.6
Gender			
Male	145	130	275
Female	47	62	109
BMI (KG/m^2)	28.4±1.1*	22.6±0.2	25.5±0.6
Smoking (%)	50.2*	19.4	19.4
Hypertension (%)	54.5*	25.2	25.2
Diabetes (%)	30.6*	10.1	10.1
Triglycerides (mg/dl)	195±2.8*	124±1.7	159±2.4
LDL-C(mg/dl)	155±1.9*	111±2.9	133±2.0
HDL-C (mg/dl)	42±1.0	50±0.8	46.3±0.6

All continuous variables are expressed in means ± standard error (SE) and categorical variables are expressed in number with percentage in parentheses. Abbreviation: LDL-C = low density lipoprotein cholesterol, BMI = body mass index, HDL-C = high density lipoprotein cholesterol, *$P < 0.05$

Table 4.4 Annealing temperature and sequencing primers used for sequencing of selected SNPs

SNP NO	SNP ID	Chr	Gene	Annealing TM (°C)	Sequencing primer
S1	rs4977574	9p21.3	*CDKN2A/B*	56	R
S2	rs2891168	9p21.3	*CDKN2A/B*	58	F
S3	rs2383206	9p21.3	*CDKN2A/B*	60	F
S4	rs2383207	9p21.3	*CDKN2A/B*	56	F
S5	rs10811656	9p21.3	*CDKN2A/B*	56	F
S6	rs10757278	9p21.3	*CDKN2A/B*	58	F
S7	rs1333049	9p21.3	*CDKN2A/B*	58	F
S8	rs10757283	9p21.3	*CDKN2A/B*	56	R
S9	rs1333047	9p21.3	*CDKN2A/B*	56	F
S10	rs10757277	9p21.3	*CDKN2A/B*	60	F
S11	rs10757279	9p21.3	*CDKN2A/B*	56	F
S12	rs3135506	11	*APOA5*	58	F
S13	rs1558861	11	*APOA5*	58	F
S14	rs662799	11	*APOA5*	60	R
S15	rs10750097	11	*APOA5*	58	F
S16	rs599839	1P13.3	*PSRC1*	60	F
S17	rs646776	1P13	*CELSR2*	56	R
S18	rs3846663	5q13.3	*HMGCR*	56	F
S19	rs2271293	16	*NUTF2*	56	F
S20	rs6511720	19	*LDLR*	60	F
S21	rs1537375	9	*ANRIL*	58	F
S22	rs4986790	9	*TLR4*	58	F

Chr = chromosome; TM = temperature; F = forward; R = reverse

4.4 Genomic DNA extraction from human blood

The concentration of genomic DNA which was extracted from blood was at least, 80 µg/ ml measured by nanodrop and also was imaged by 1.0% agarose gel. Extracted DNA samples were shown in Figure 4.5.

Genomic DNA of different samples in 1% agarose gel

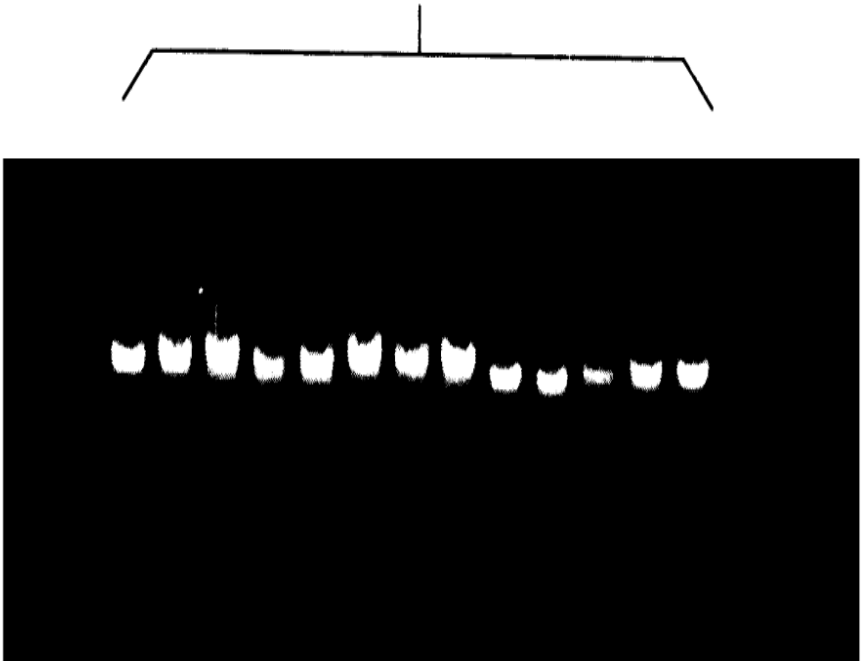


Figure 4.5: Genomic DNA extracted by whole blood

A total of 9 genes were carefully selected for analysis selected from extracted DNA based on the reported genetic association with MI from NCBI database, 22 SNPs were identified and evaluated for the association with MI. As an example one gene indicating the region of SNP location with first and nested PCR primers are as follows (Figure 4.6).

db SNP: rs10757278

Find in NCBI database (Fasta)

AATAAATATTGTATTAGTTCTCATTTTGTAAAACTTATACAGGTATCATATGCATAGAC
AAATACACCAAACTGATGAATATTTGCCTTGTATAATCTTTTTGTAGTTTTTTTATGAACATATA
TTACTCAAACAATTTAGAACATTTGGCAATATATATATATTTCATTTATAAAAGGTTAGGAAGAT
TAATTACACTTTCTGAGGTCGCAACTAAAAGCCAAGATTTTAATCCATTTCTATTTGATGTAAAG
TCTGGTCTTTTTTCAGCAAACCACAATCCCACATTTTAAGGGCATTAAGAAAGGGATGGGTAGAC
AAAATGTAGAGGTAGTAGGTACAGAATACAAAGTTTCAAGAAATTAAAAGCTTCTAAACTAACAA
ACAGCCAATTTGTGGAGTGTCACTGGAAAGTGACAAAGAGGACAGTTAAGTTAGTTGGAACTGAA
CTGAGGCCAGACAGGGCTGTGGGACAAGTCAGGGTGTGGTCATTCCGGTA**R**GCAGCGATGCAGAA
TCAAGACAGAGTAGTTTCTCCTTCTCTCTCTCTCTTTAATTGTAACGCCTTTTATAACAAACAAA
TATTATGCTTATTTCTGTCTTTAAATTTTTTGTAGTAATTTCTCATCACTTAACCTCTATTTTTT
AAAAAACTAACTTTTCTCTTGTTTTTCTAGTTGAGCTATCATTCATATTTATTATGTGGAACTAG
AGGTAGTCCTGGCTACTTGGGAACAGCGTGGAGTCTAGCCATGTCAGGGCCAGAAGTCGTCTCAG
CTAAGTTAGAATGTGATACCATTGTTTACACAAGTGTGGCCTGCCTTCAAGATAGGGTGAGGTGT
TTTATGACCACAGGCTTTATGAGTTATAGCTATAAAACAATCAATCTTTTAAAGCAAACACACCA
CAATGTCTACACGCAAATGAGAATTGTCCTTCAGGGCTGTGACCTTGGGAAACTCTTCACACATA
CTAGCATAAAACATATTTAAACTCTTCACTG

FIND IN UCSC (dB SNP: rs10757278)

TTTCCTACTCTTTAATTAGATTGTTTTTACTGTTGAGATGTTTGAGTCCCTTATATATT
CTAGATATTAATCTCTTGTCAGATGAGTAGTTTGCAAATATTTTCTTCCATTCTGTAGTTTGTAT
ATTCACTCTGTTGATTACTTCCTTTGCTGTACAAAAGCTATTTGGATTGATATAATCCCATTTGT
GTATTTTGTTTGTGTTACCTATGTTTTTAAGGTATTATTCAGAAAATTTTTGCCCAGACCAAGGT
CCTGAAGCATTTCTTCTATGTTTTCTTCTAGTAATTCTATTTTTCATGTCTTACATTTAGGTTTT
TGATCCATTTTGCAATGATATTTATATAGGGTGAGAGACGGGAATCTAGTTTAATTCTTCTGCAT
ATAGATATCCAGTTTTCCCAGCACCATTCGTTGAGGAGACTGTCCTTTACCCACTGAGTGTTCTT
GATATCTTTGTTAAAAATCAGGTGGTTGTAGATATGTGGATTAATTTCTGGGTTCTCTATTCTGT
TCTATTGATCTATGTGTCTGTTTTTATACCAGTACCATGCTATTTTGTTTACTATAGCTTTGTAG
TATACTTTGATGTCTGGTAGTGTGATAACTCCAGCTTTGTTCTTTTTGCTTAGGATTGCTTTGGC
TGTTTGGGGTCTTTTGTGGTTCCATATAAATTTTAGAATTTTTTTTTCTATTTCTGTGAAGAACG

TCATTAGTATTTTGATAGGGATTGCATTGAATCTATAGGTTGCTTTGGGCAGCAGATATCTTTCC
ATTTGTTTATGTCCTTTTCCATTTCTTTCATCAGTATTTTGTAGTTTTAGTTGCAGAGGTCTTTC
ACCTCCTTGGTTACATTTATTCCTTGGTATTTTATTGTTTTCGTAGCTATTGTAAATGGGATTGC
CTTCTTGATTGCTTTTTCAGCTAGTTCATTGTTCATGTATAGAAATACTACTGATTTTTGTATAT
TGATATCGTATTCTGCAACTTTACAGAATTTGTTATTAGCTCTGAGTTTTTTGGTAGAGTCATTA
GATTTTTCTGTATATAAAATCATGTCATCTGCAAACAGGAACAATTTGACTTCCTCCTTTCCAAT
TTGGATGTCCTTTATTTCTTTGTCTTGCCTAGTTGCTTGGGCTAGGACTTCCATTACTGTGTTGA
ATAAGAATGTTAAGAGTGGGCATCCTTGTCTTGTTTCAGTTCTTAGAGGAAATGCTAGTGTAGCT
GAGGAGAGAAATGAAAACAGAATAAAAACTTCAGTAGAAGTTGGGGAGAAATGTGTTTGCACCAA
AAGAGGTGAGGGATGGAACTGAGGGAGGTCTCGAGGGGTAAGAGAGGCCAAAGAGCTGGC**AGGTG**
CTGCTATTAGGATGGCCAATGATCCAGAAGTAATGATGAATGAGAAGATGGATTAAAAAAACCCT
GACATTGTTTAACTAGAAGAAGAAGACAGTCAGAGAGAAGTGAGGGCTTACTTTTCATGTTTAAA
GTCTGTTATGTGGTAAAGGGATTAGATTTATCTGTGTTGTTCCAGGGGACAGAAATAGGACAAAT
GGATGCAAATAGAGTGAGGAAGATTTAAAACAAATGGAGAAGACATTCTAAAATCAACTACAATG
AGCGTAAACAATGACAACGGAAAGGACTATTTTTGCAATTATGTAGCTCTTGTTCTCTATAGATA
GATGTGTAAGCAGACGCTGAATAACCATTTACTGAAAATATAAATAAAAACAAAGCAAATTGCAG
GCAATAAATATTGTATTAGTTCTCATTTTGTAAAACTTATACAGGTATCATATGCATAGACAAAT
ACACCAAACTGATGAATATTTGCCTTGTATAATCTTTTTGTAGTTTTTTTATGAACATATATTAC
TCAAACAATTTAGAACATTTGGCAATATATATATATTTCATTTATAAAAGGTTAGGAAGATTAAT
TACACTTTC**TGAGGTCGCAACTAAAAGCCA**AGATTTTAATCCATTTCTATTTGATGTAAAGTCTG
GTCTTTTTTCAGCAAACCACAATCCCACATTTTAAGGGCATTAAGAAAGGGATGGGTAGACAAAA
TGTAGAGGTAGTAGGTACAGAATACAAAGTTTCAAGAAATTAAAAGCTTCTAAACTAACAAACAG
CCAATTTGTGGAGTGTCACTGGAAAGTGACAAAGAGGACAGTTAAGTTAGTTGGAACTGAACTGA
GGCCAGACAGGGCTGTGGGACAAGTCAGGGTGTGGTCATTCCGGTA**A**GCAGCGATGCAGAATCAA
GACAGAGTAGTTTCTCCTTCTCTCTCTCTCTTTAATTGTAACGCCTTTTATAACAAACAAATATT
ATGCTTATTTCTGTCTTTAAATTTTTTGTAGTAATTTCTCATCACTTAACCTCTATTTTTTAAAA
AACTAACTTTTCTCTTGTTTTTCTAGTTGAGCTATCATTCATATTTATTATGTGGAACTAGAGGT
AGTCCTGG**CTACTTGGGAACAGCGTGGA**GTCTAGCCATGTCAGGGCCAGAAGTCGTCTCAGCTAA
GTTAGAATGTGATACCATTGT**TTACACAAGTGTGGCCTGCC**TTCAAGATAGGGTGAGGTGTTTTA
TGACCACAGGCTTTATGAGTTATAGCTATAAAACAATCAATCTTTTAAAGCAAACACACCACAAT
GTCTACACGCAAATGAGAATTGTCCTTCAGGGCTGTGACCTTGGGAAACTCTTCACACATACTAG
CATAAAACATATTTAAACTCTTCACTGAAAATATTATTCAGACCTAGTTTCTCTCTCAGTCTCTC
TCTATGTTTCTCTCTCACATGTACGTGCATGCACGCACACGTGTGCACACACACATATGCTCACA
TCATTTTAAGATACATCTCATTTTTAACCAAAACCATTTTATCTTGCTTGATAACCAATTTTATT

TGTTAGATGACTTGGCTATAAATGCCTTTGGCTATCAGGAAAATCAAATCAAAATTAAAAGACAC
TGTTACTACTGAAGAAGTAAAAAAAGAATGGGCTGCTGACTCTGAAGATCATACCCGAAGTAGAG
CTGCAAAGATATTTGGAATATTGGTAATATCCAATAAAGAATGACCTTCATGCTATTTTGAGGAG
ATGTTTAAATGTCGAATTATTGAAATATTTATAAAATACAAATAAACTAACTCTGCTTCATATTC
CAACTTGTGTATGACACTTCTTAGGCTATCATTTCATTCCAAATTTATGGTCACTACCCTACTGT
CATTCCTCATACTAACCATATGATCAACAGTTGAAAAGCAGCCACTCGCAGAGGTAAGCAAGATA
TATGGTAAATACTGTGTTGACAAAAGTATGCAGAAGCAGTCACATTTATACAGTAGTGAAGGAAA
TGTAAATTGGACAAACTTTTTGGAAGATAAGTTGAGAATGTCAAAAATCAAAACACACTTTCTGT
TTTATTCAGCAATTATGAGCCCTTTGTTTTACAGCTATGCTCACAAATATATACAAACATGTATG
CACAATTATGTTCACTGTGGTATTGCGCTAGAAAAATACTAAAAACAAACCAAATGTTCATCAAT
AGGGAAATTGTTCAACAAATTACAGTATATCTAAAAAAAGAATAATATATAACAACTGAAAAAAT
AAAATAGTTGATATAAGCAGATATTCCAAGATCTGCCAGACATATTGTTAAACGAAAAATCTAGA
TACAAAATTGTTTATAGTTCTCTTTCATACTATAGCCAAAGAAAATTCAGAAAAAACTACTTACA
GTTGATCCTTGAATAATGCAGCAGGTAGGGGCACCAGCTTCCTGCGCAGTCAAGAATTGCATATA
ACTTTTGACTCCCCCAAAACTTTGCTAATAGCCTACTGTTGACTGGAAGCCTTATCAACAACATC
AACAGTTGATTAACACTGGAAAAGGAGGGGTTGGTCTTACTGTCTCAAGGGTGGCAAAGGCGGAA
GAAAAGCCACCTATAAGTGAACCCTTGAAGTTCCAACCTATGTTTTTCCAGGGCAATCATACATC
CATGTCCATATTCATGATGAATATGTGAAAGGGTACATAAGAAAATGTGATAGTCGTTTCCTCTG
GAGAATAAAATGAGAGGTCTATCATAGGTGAGAAAATAATTTTTTATTATATATTCTTTTGTAGG
TTTATTTTTTTGTCATGTGTATGTATTCTTTTCAAAATAATAAAAACAATAACAACGATTAAAAT
ATACAAGGGGAAATGCATTGGCCAAGGTTTAGTTGAGAGCAAAGTTTGCTTATCTTTCGAAAGAG
GTGAACTAAGCTTTTCCTTTTTCTCTTTGGGTATACTTGAATGGGGATGGAGTAAGTGGATTCTT
TTTTTTTTTTTTTTTTTTTGAGACGGAGTCTCGCTCGCCCAGGCCGACTGCAGTGGCGCTATCTC
GGCTCACTGCAAACTCCGCCTCCGGAGTTCACGCCATTCTCCTGCCTCAGCCTCCCGAGTAGCTG
GGACTACAGGCGCCCGCCACTG

Figure 4.6: Part of data base sequence of gene *CDKN2A/2B* (rs10757278). Yellow color represents the 1st PCR product while the ferozy color represents the nested PCR product. While red colour in NCBI and UCSC sequence representing SNP location for gene *CDKN2A/2B* (rs10757278)

4.5 Nested PCR product

After the extraction of genomic DNA, all samples were amplified by nested PCR. The PCR products were imaged by 1.0% agarose gell electrophoresis. Results of nested PCR products for 11 genes are shown in Figure 4.7 (a-i) with 1kb DNA marker.

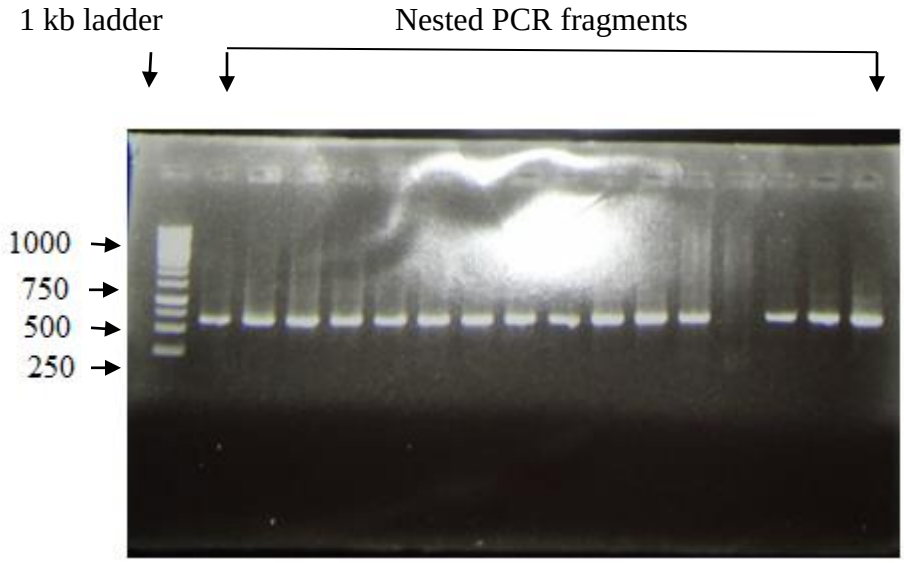


Figure 4.7 (a): Nested PCR product for gene *CDKN2A/2B* (rs10811656) with product size = 559 visualized in 1% agarose gel

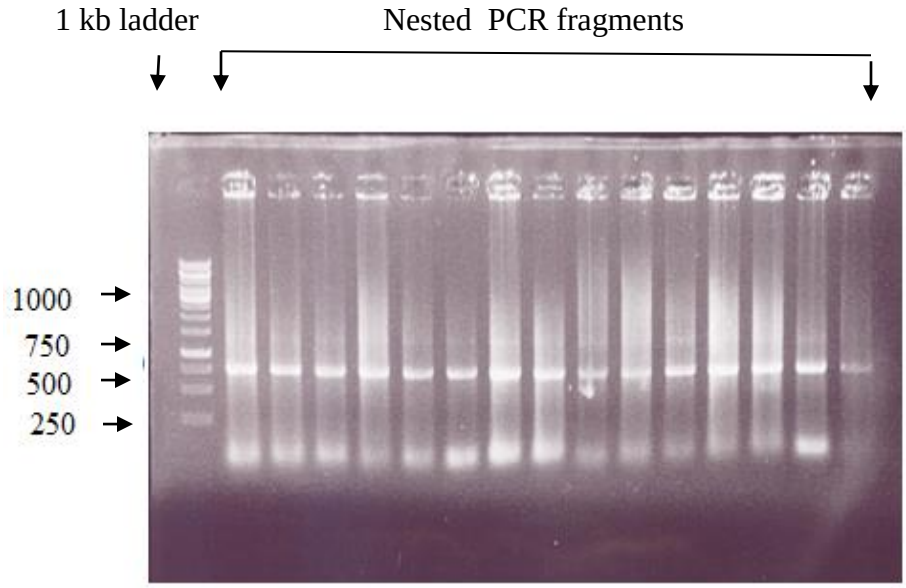


Figure 4.7 (b): Gene *APOA5* (rs3135506) product size = 650

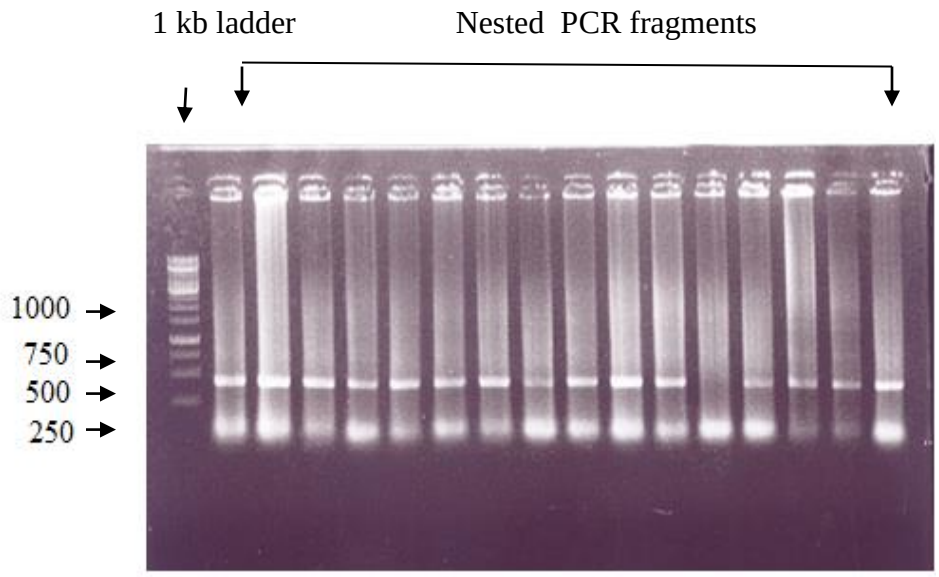


Figure 4.7 (c): Gene *PSRC1* (rs599839) product size = 539

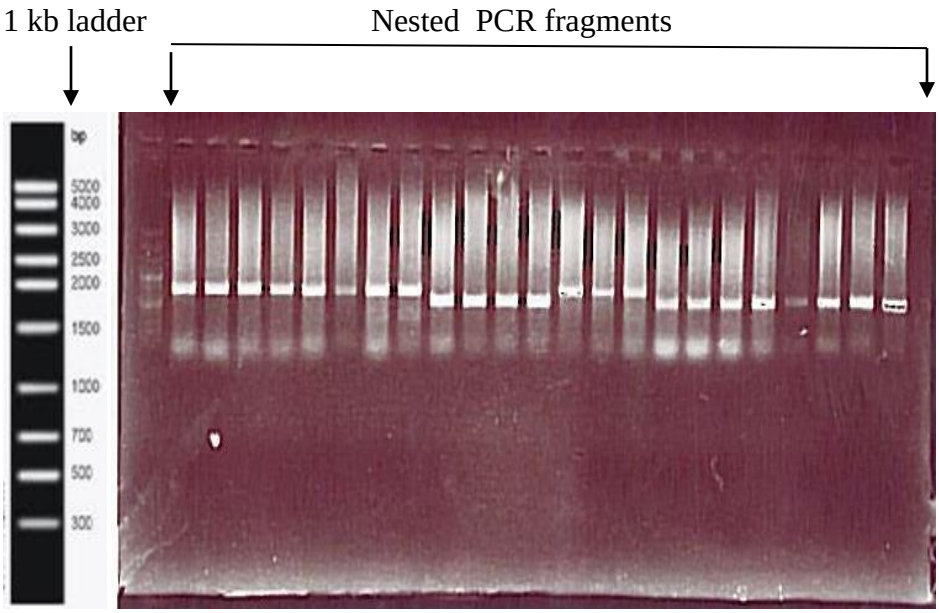


Figure 4.7 (d): Gene *CELSR2* (rs646776) product size = 560

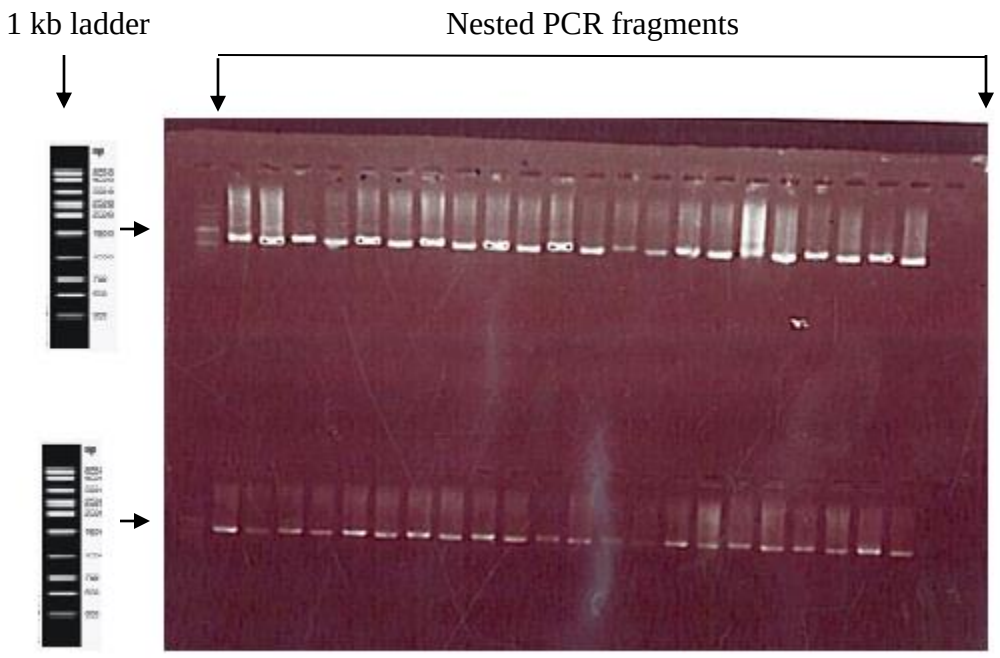


Figure 4.7 (e): Gene *HMGCR* (rs3846663) with product size 698 bp

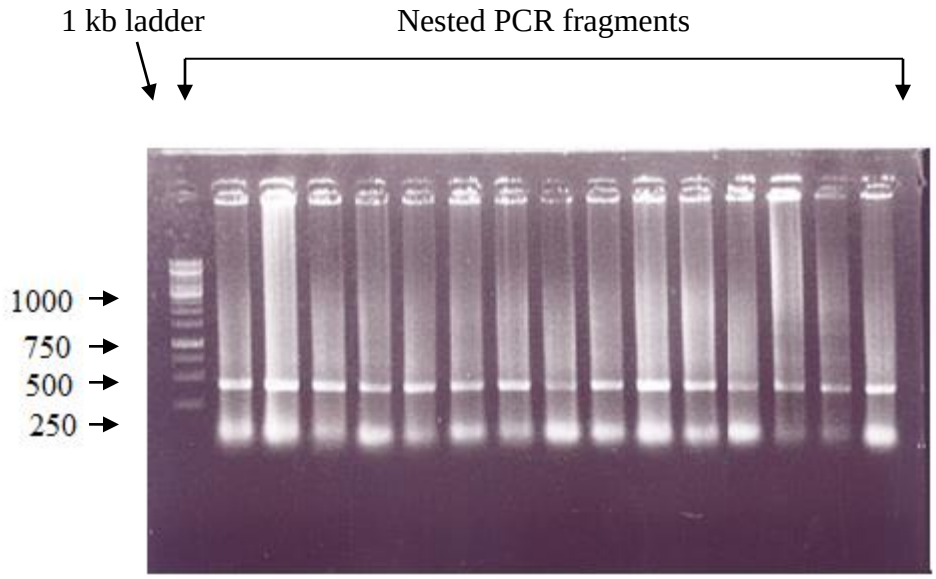


Figure 4.7 (f): *NUTF2* (rs2271293) with product size 529 bp

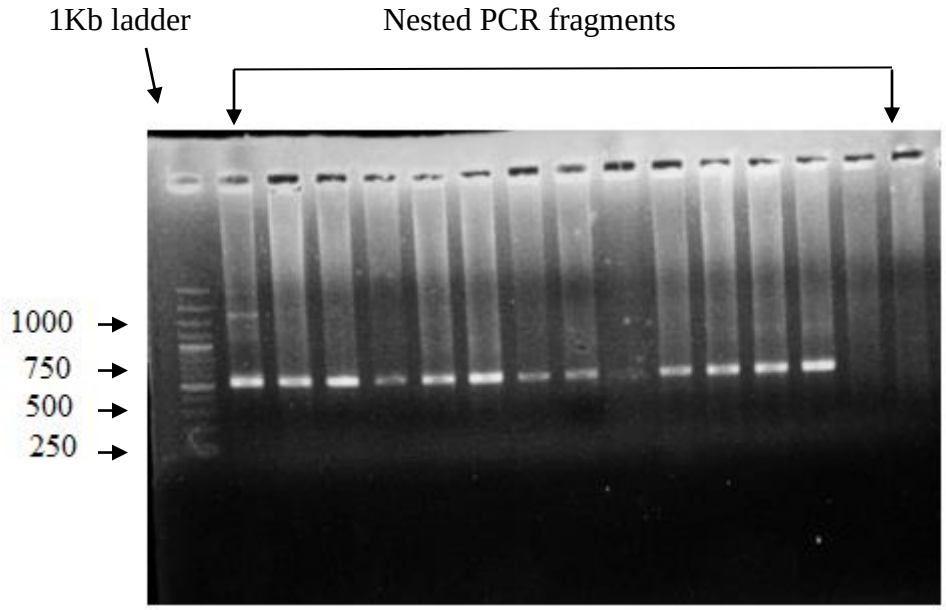


Figure 4.7 (g): *LDLR* (rs6511720) with product size 669bp

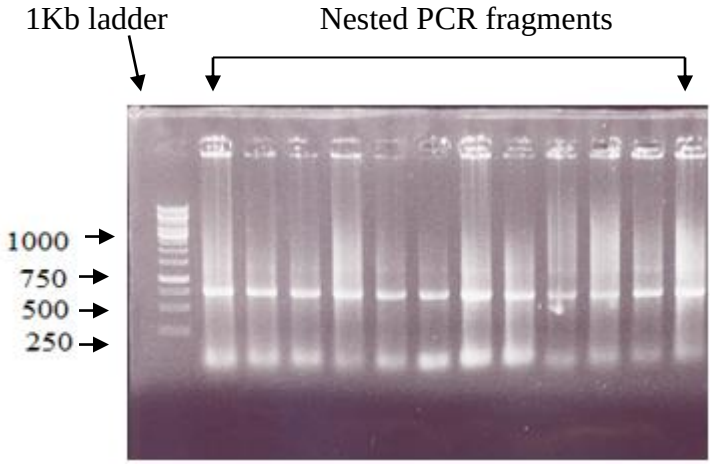
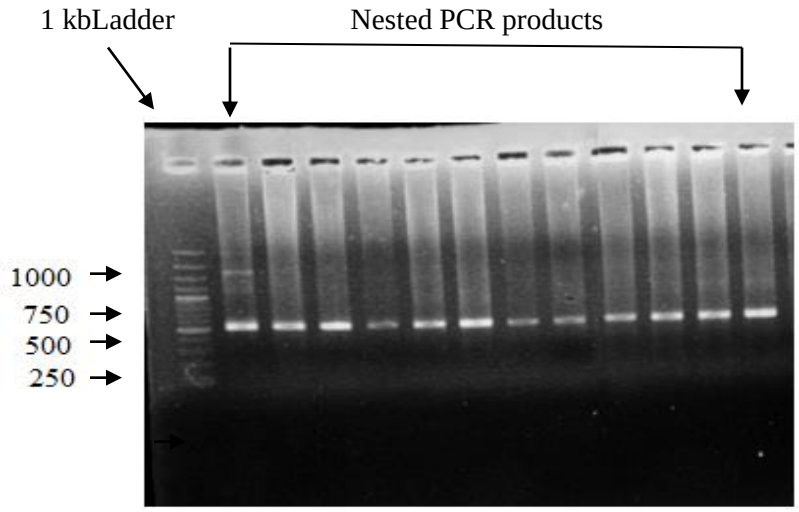
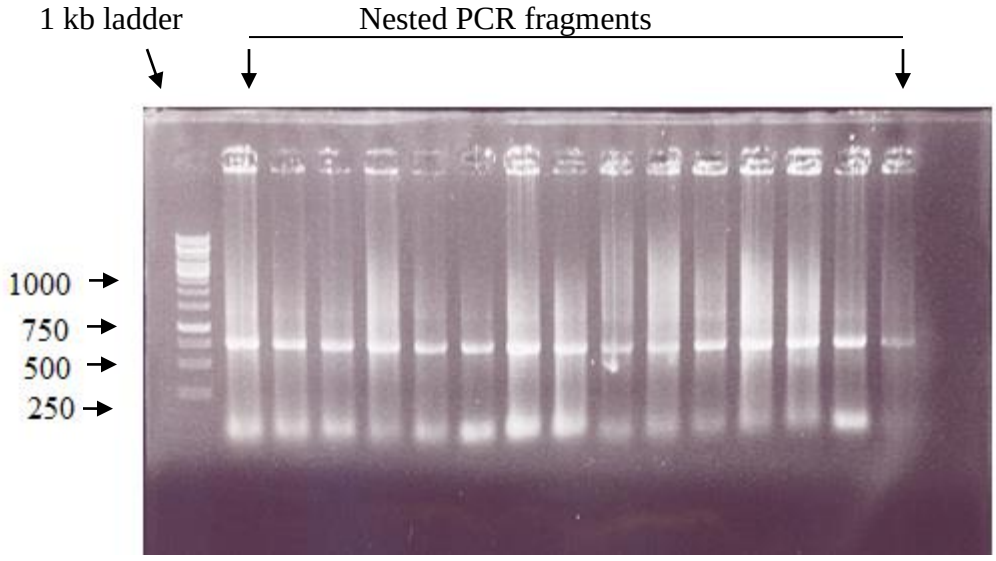


Figure 4.7 (h): *ANRIL* (rs1537375) with product size 750 bp



(i) Gene *LDLR* (rs6511720) with product size 669 bp



(j) Gene *TLR4* (rs4986790) with product size 690 bp

Figure 4.7 (a-j): Nested PCR products visualized in 1% agarose gel

4.6 Recognition of SNP site

Chromatograms (Figure 4.8) are showing the SNP sites within the part of nucleotide sequence. These chromatograms are representing three genotypes; homozygous dominant, heterozygous and homozygous recessive identifying by arrow in DNA sequencing for each database (dbSNP). There were three SNPs (rs6511720, rs1537375 and rs4986790) out of 22, having only one genotype (homozygous) in cases and control (Figure 4.8 p, q, and r).

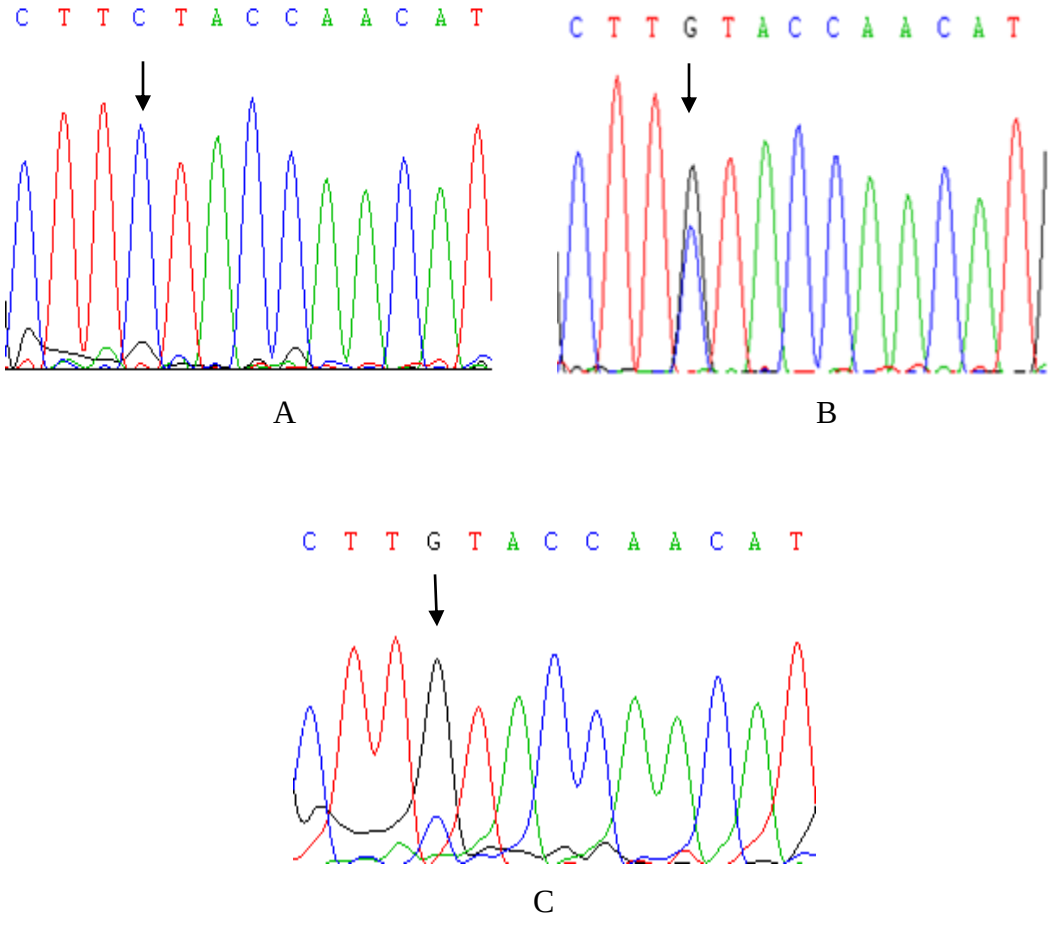


Figure 4.8 a: Part of nucleotide sequence of rs1048990 SNP *(CDKN2A)*. (A) CC genotype; (B) CG genotype; (C) GG genotype

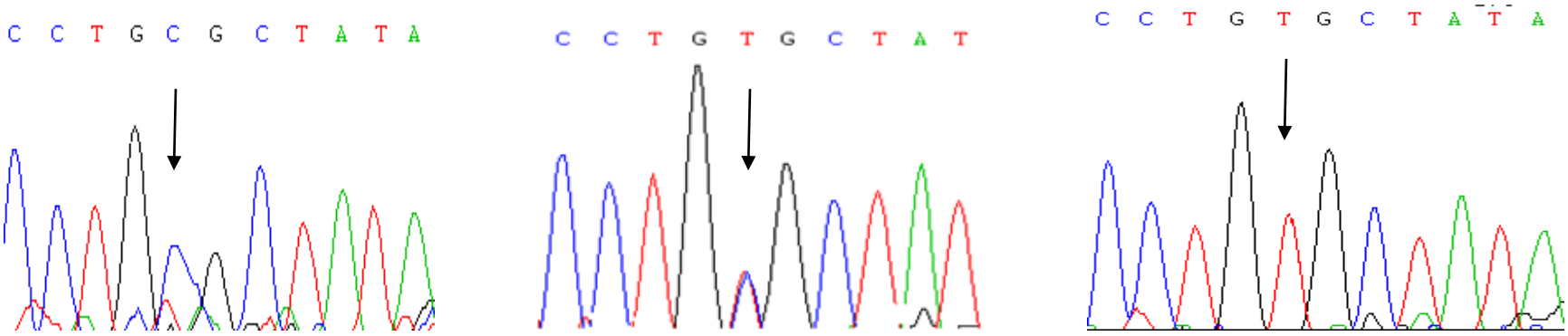


Figure 4.8 b: Part of nucleotide sequence of rs4977574 SNP *(CDKN2A)*. (A) CC genotype; (B) CT genotype; (C) TT genotype

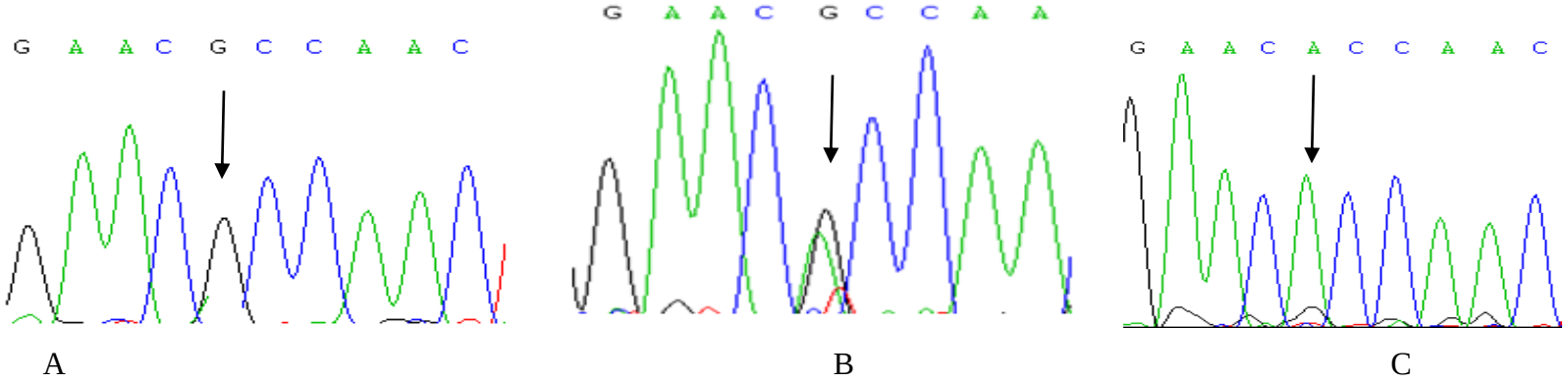


Figure 4.8 c: part of nucleotide sequence of rs2891168 SNP *(CDKN2A)*. (A) GG genotype; (B) AG genotype; (C) AA genotype

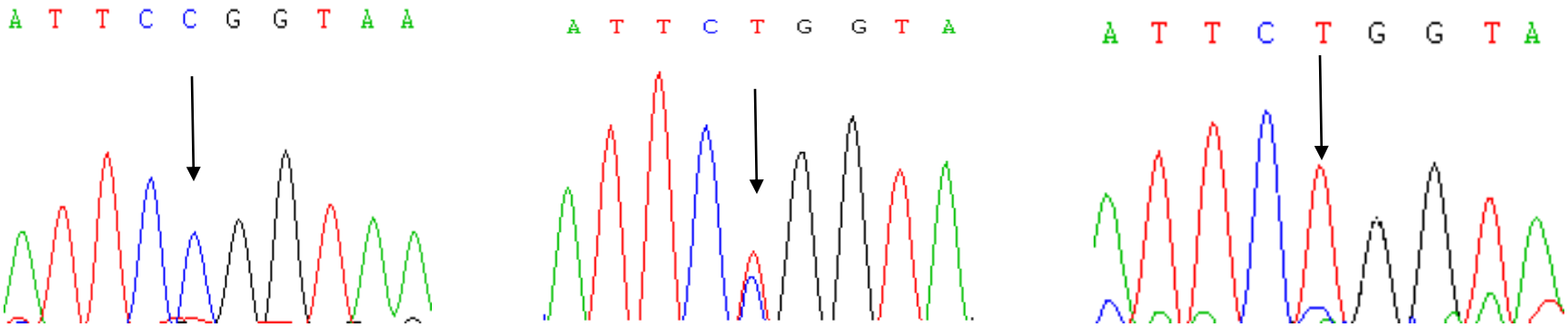


Figure 4.8 d: Part of nucleotide sequence of rs10811656 SNP *(CDKN2A)*. (A) CC genotype; (B) CT genotype; (C) TT genotype

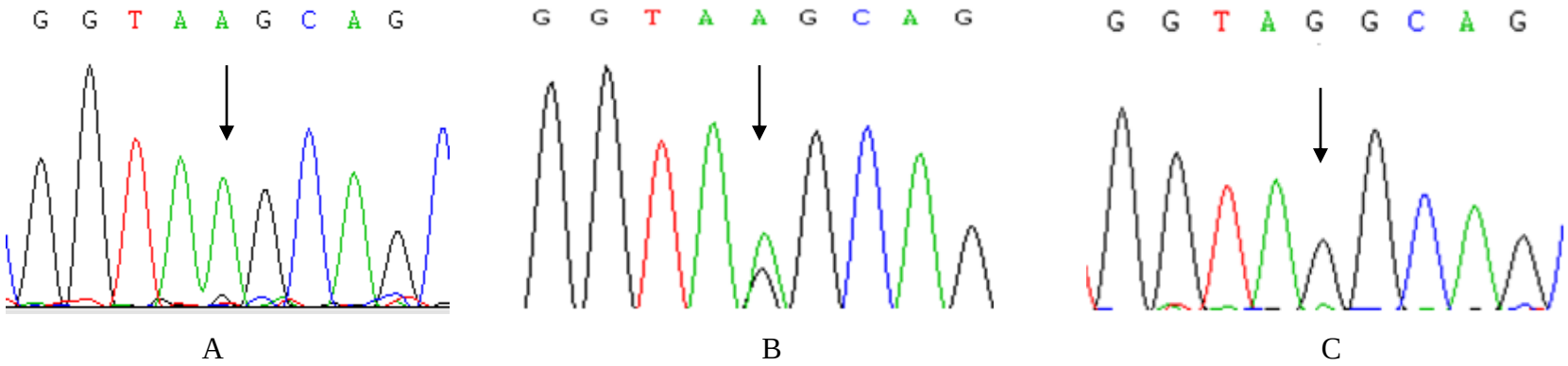


Figure 4.8 e: Part of nucleotide sequence of rs10757278 SNP *(CDKN2A)*. (A) AA genotype; (B) AG genotype; (C) GG genotype

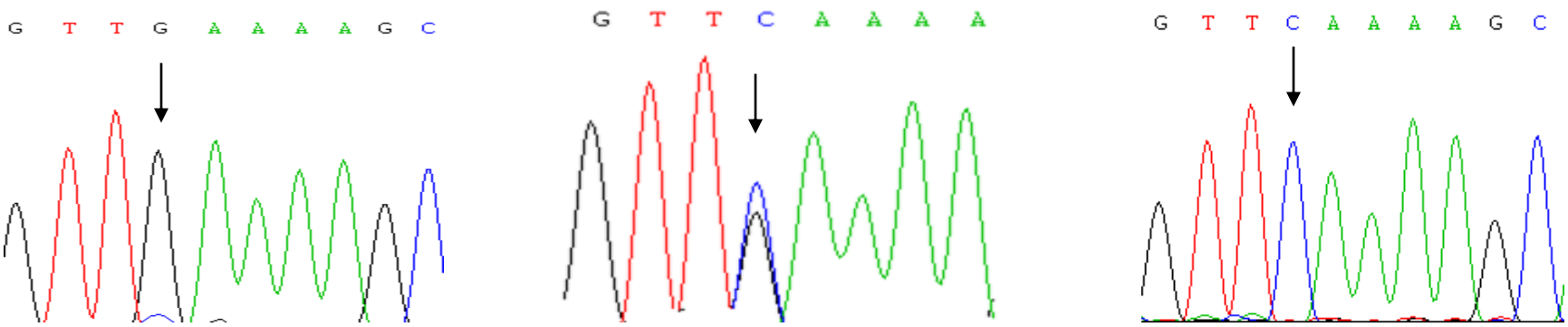


Figure 4.8 f: Part of nucleotide sequence of rs1333049 SNP *(CDKN2A)*. (A) GG genotype; (B) GC genotype; (C) CC genotype

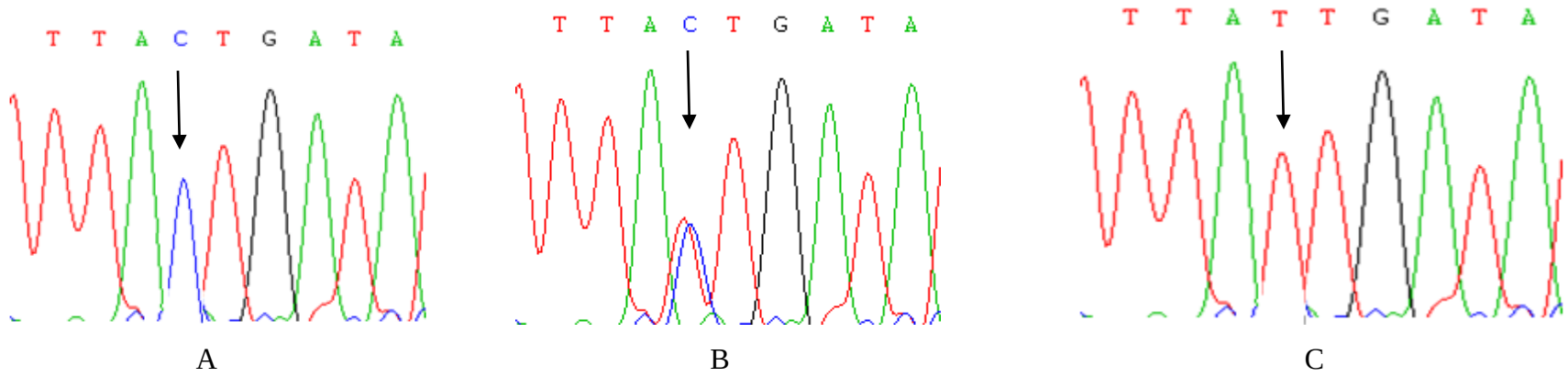
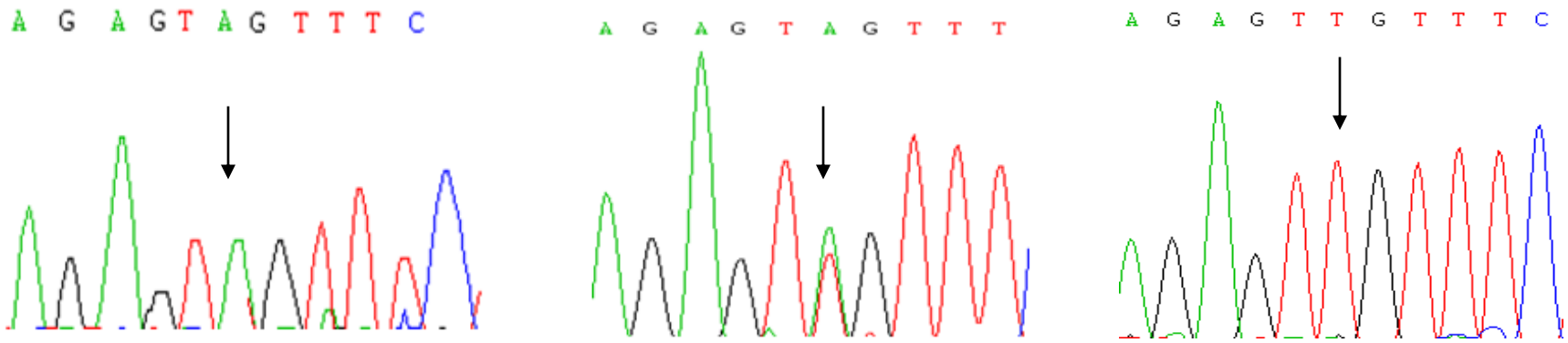
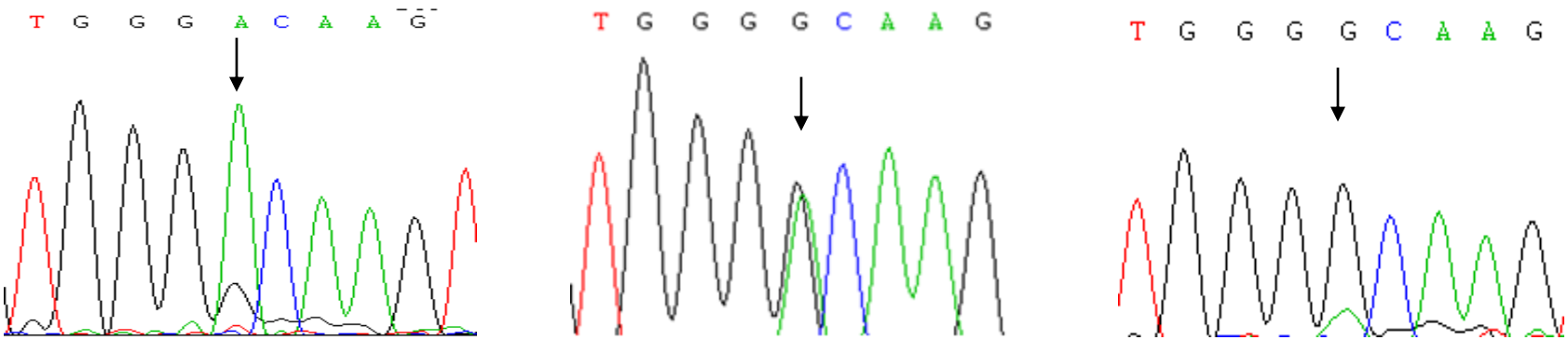


Figure 4.8 g: Part of nucleotide sequence of rs10757283 SNP *(CDKN2A)*. (A) CC genotype; (B) CT genotype; (C) TT genotype



A B C

Figure 4.8 h: Part of nucleotide sequence of rs1333047 SNP *(CDKN2A)*. (A) AA genotype; (B) AT genotype; (C) TT genotype



A B C

Figure 4.8 i: Part of nucleotide sequence of rs10757277 SNP *(CDKN2A)*. (A) AA genotype; (B) AG genotype; (C) GG genotype

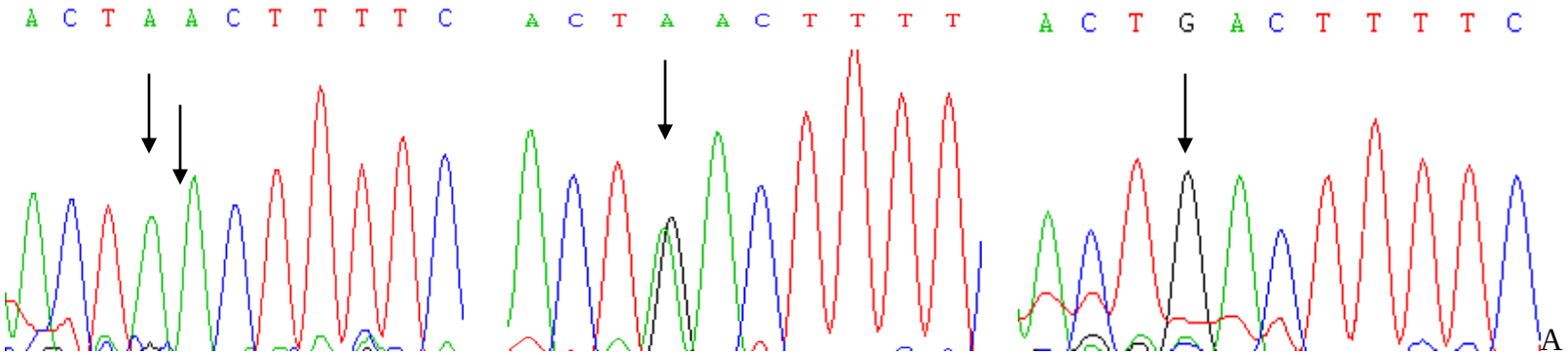


Figure 4.8 j: Part of nucleotide sequence of rs10757279 SNP *(CDKN2A)*. (A) AA genotype; (B) AG genotype; (C) GG genotype

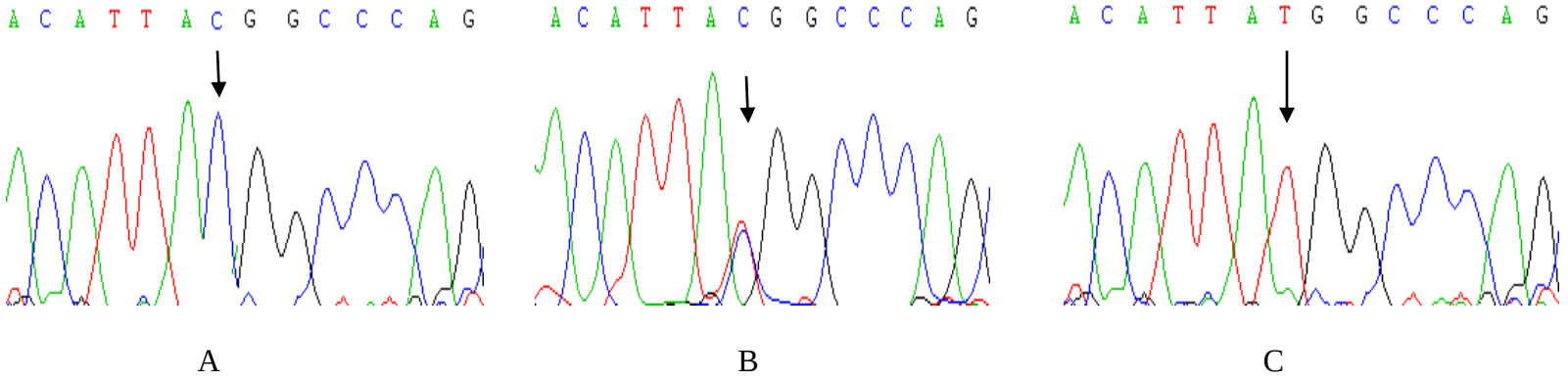
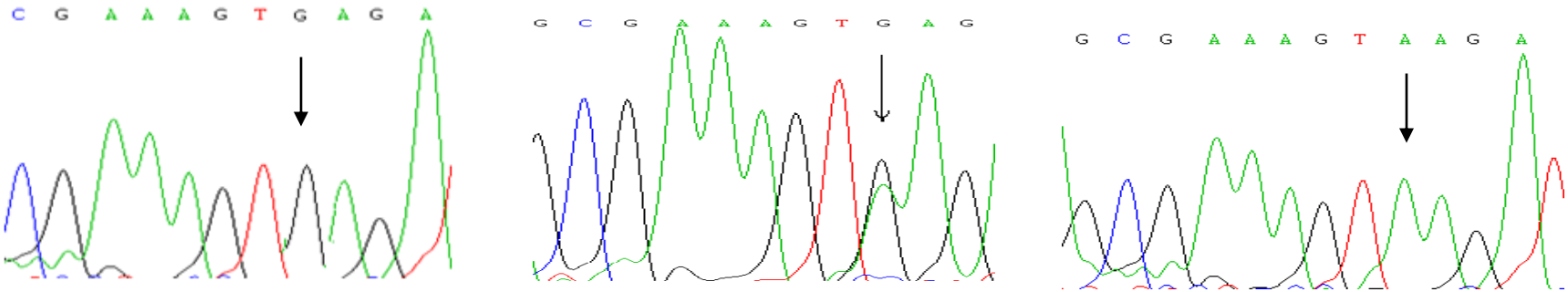
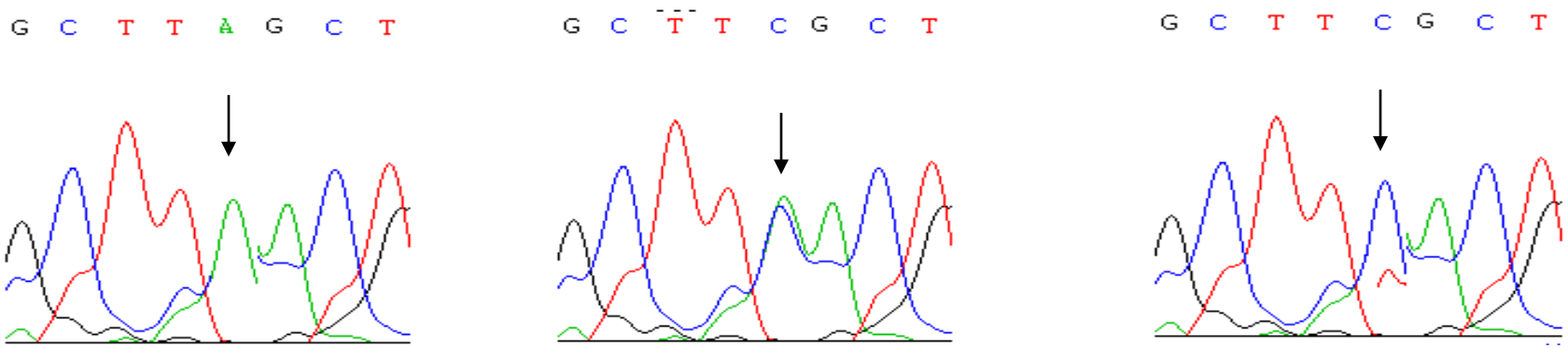


Figure 4.8 k: Part of nucleotide sequence of rs1558861 SNP *(CDKN2A)*. (A) CC genotype; (B) CT genotype; (C) TT genotype



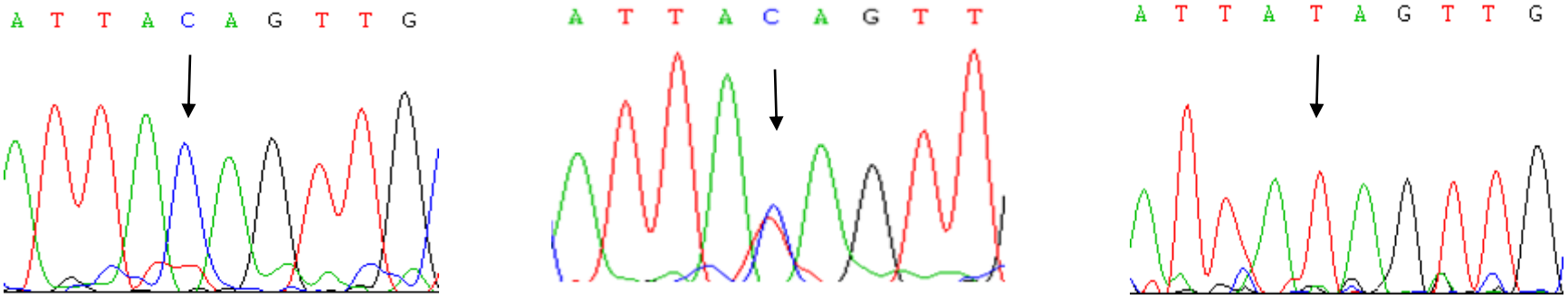
A B C

Figure 4.8 l: Part of nucleotide sequence of rs662799 SNP *(CDKN2A)*. (A) GG genotype; (B) AG genotype; (C) AA genotype



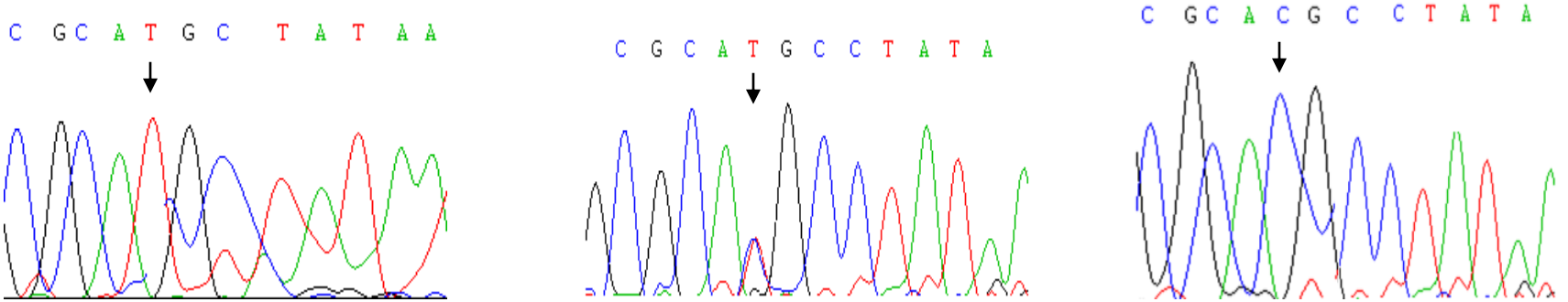
A B C

Figure 4.8 m: Part of nucleotide sequence of rs4665058 SNP *(CDKN2A)*. (A) AA genotype; (B) AC genotype; (C) CC genotype



A B C

Figure 4.8 n: Part of nucleotide sequence of rs3846663 SNP *(CDKN2A)*. (A) CC genotype; (B) CT genotype; (C) TT genotype



A B C

Figure 4.8 o: Part of nucleotide sequence of rs3918242 SNP *(CDKN2A)*. (A) TT genotype; (B) CT genotype; (C) CC genotype

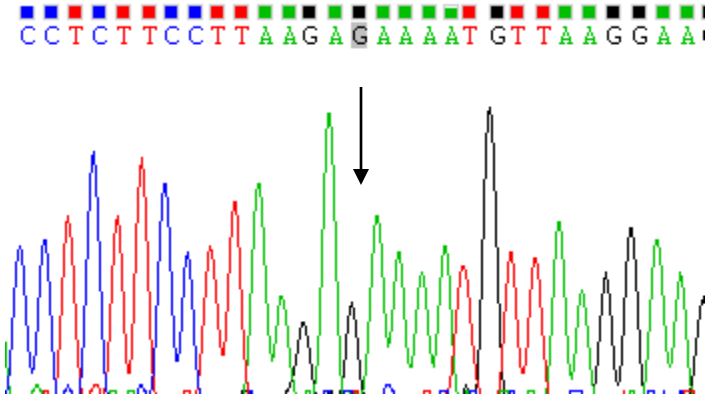


Figure 4.8 p: Sequence of rs6511720 SNP *(LDLR)* GG genotype.

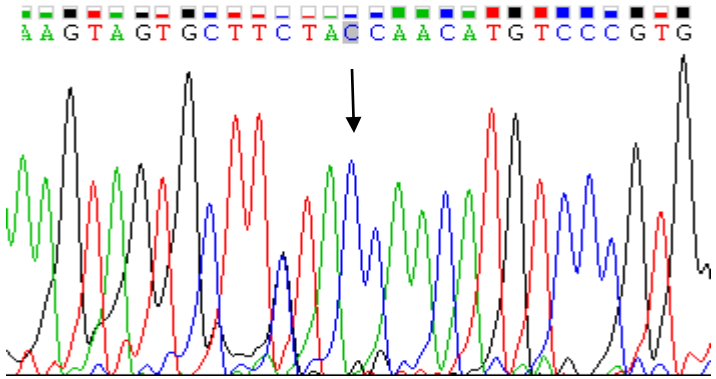
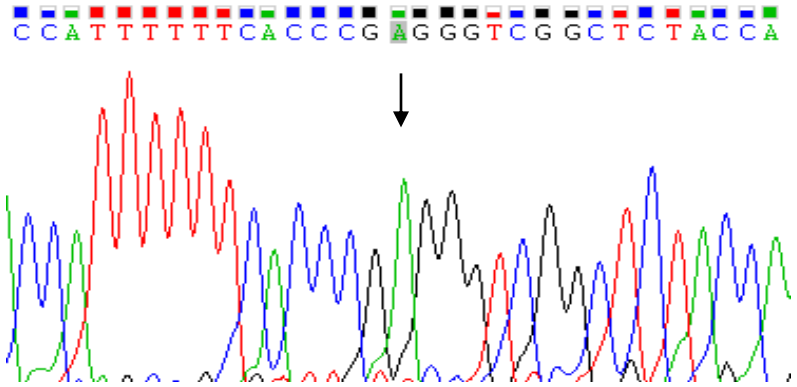


Figure 4.8 q: Sequence of rs1537375 SNP *(ANRIL)* CC genotype



. **Figure 4.8 r:** Sequence of rs4986790 SNP *(TLR4)* only AA genotype was found

4.7 OLE test and OLE assay analysis

OLE test results of one SNP (rs10757278) are shown in Annexure V. According to FP readings from multilable counter of OLE test (tempelate generation), that method (one out of four methods) was selected for OLE assay which depicted higher value of homozygous genotype. Results with base line value of OLE assay for one SNP rs10757278 are described in Annexure V.

4.8 Association of candidate genes

As described in section 4.2, out of 22 SNPs 19 were selected for disease association analysis and were in HWE (HWE exact test $P > 0.05$) in the control group. After HWE test, all 19 genetic variants were included for disease association analysis.

4.8.1 Allelic associations of 9p21 locus with MI

The *CDKN2A/B* gene (chromosome 9p21.3) was sequenced for 11 SNPs and out of them only 8 were strongly associated with onset of MI ($P \leq 0.05$). In case of allele level significant associations with MI at chromosome 9 were found at following genetic variants: rs4977574 (S1) risk allele G with odd ratio 1.394 (1.09,1.90) ($P = 0.037$), rs2891168 (S2) risk allele G ($P= 0.016$) with OR 1.48 (1.09,2.00), rs2383206 (S3) risk allele G ($P = 0.02$), rs2383207 (S4) risk allele G ($P = 0.0002$), rs10811656 (S5) risk allele T ($P = 0.0012$), rs10757278 (S6) risk allele G ($P = 0.005$), rs1333049 (S7) risk allele C ($P = 0.02$) and rs10757283 (S8) risk allele T ($P = 0.01$). While three SNPs, rs1333047 (S9), rs10757277 (S10) and rs10757279 (S11) of 9p21.3 locus were not significantly ($P > 0.05$) associated with disease (Table 4.5 and 4.6).

The SNP rs10811656 and rs10757278 from *CDKN2A/B* ($P = 0.006$ and 0.004 respectively) were with three genotypes (CC, CT and TT and AA, AG and GG) and their minor alleles TT and GG were found with greater frequency (0.13 and 0.08) in patients than control (0.07 and 0.03). The studied SNPs were strongly associated with MI onset (Table 4.7).

4.8.2 Allelic associations of lipid metabolism related SNPs with MI

Further we analyzed 8 SNPs (chromosomes 1, 5, 11 and 16 respectively) previously reported to be associated with LDL-C, HDL-C, triglycerides and CHD from GWAS. A significant association of these genetic variants was determined with MI. The 4 SNPs (S12, S13, S14 and S15) were located in *APOA5* gene, 2 SNPs (S16 and S17) were found in *CELSR2/SORT1/PSRC1* gene, 1 SNP (S18) in *HMGCR* gene and 1 SNP (S19) in *NUTF2* gene. Genetic variants of both *APOA5* and *CELSR2* genes were strongly associated with onset of disease.

The minor alleles of *APOA5* SNPs rs3135506 (S12; allele C), rs1558861 (S13; allele C), rs662799 (S14; allele G) and rs10750097 (S15; allele G) were more significant ($P < 0.05$) among MI group than control. Two SNPs: rs599839 minor allele G ($P = 0.009$) and rs646776 minor allele G ($P = 0.001$) depicted strong association with disease and were located on the same chromosomal region 1p13.3 (*PSRC1/ CELSR2*). Minor allele frequencies of all selected SNPs in MI patients compared with control were shown in Table 4.5and 4.6.

The gene *HMGCR* is located on chromosome 5 and one SNP (rs3846663) was targeted on this locus. The odd ratio for variant rs3846663 (S18) (Table 4.6) was 1.72 with 95% CI (1.27-2.32). One SNP rs2271293 (S19) from *NUTF2* gene have no any impact for risk of MI. Upon comparison of the genotypes of SNPs obtained with OLE assay for all 384 samples with those scored by direct DNA sequencing 100% agreement was found reconfirms the results for SNP genotyping. Genotype frequency differences were also analyzed. Overall significant genotype associations with MI was observed for variants; S2, S3, S4, S5, S6, S8, S12, S13, S14, S15, S16 and S17 (Table 4.7).

Table 4.5 Disease association of all SNPs by UNPHASE program

			Minor allele frequency (%)		
SNP NO	**SNP ID**	**Variation M/m**	**control (n=192)**	**Case (n=192)**	***P* value**
S1	rs4977574	A/G	26	33	0.037*
S2	rs2891168	A/G	32	41	0.016*
S3	rs2383206	A/G	26	33	0.022*
S4	rs2383207	A/G	26	39	0.0002*
S5	rs10811656	C/T	25	35	0.0012*
S6	rs10757278	A/G	24	34	0.005*
S7	rs1333049	G/C	38	46	0.02*
S8	rs10757283	C/T	27	35	0.01*
S9	rs1333047	A/T	34	32	0.53
S10	rs10757277	G/A	35	37	0.4
S11	rs10757279	G/A	35	34	0.801
S12	rs3135506	G/C	26	33	0.033*
S13	rs1558861	T/C	26	34	0.02*
S14	rs662799	A/G	20	28	0.03*
S15	rs10750097	A/G	27	41	0.0001*
S16	rs599839	A/G	47	56	0.009*
S17	rs646776	A/G	33	44	0.001*
S18	rs3846663	C/T	32	45	0.0003*
S19	rs2271293	G/A	30	32	0.8

M = major allele; m = minor allele, * = significant ($P < 0.05$), Adjusted *P* value from permutation test = 0.009901; standard error = 0.009901

Table 4.6 Single nucleotide polymorphisms associated with myocardial infarction

SNP	SNP_ID	HWE	RR(95% Cl)	OR(95% Cl)	†*P* value
S1	rs4977574	0.26	1.26 (1.01,1.58)	1.394(1.09,1.90)	0.039*
S2	rs2891168	0.86	1.28(1.06,1.56)	1.48 (1.09,2.00)	0.012*
S3	rs2383206	0.71	1.29 (1.03,1.60)	1.43 (1.05,1.96)	0.022*
S4	rs2383207	0.34	1.48(1.20,1.83)	1.79 (1.32,2.43)	0.0002*
S5	rs10811656	0.32	1.43 (1.15,1.79)	1.67 (1.22,2.29)	0.0015*
S6	rs10757278	0.33	0.85 (0.78,0.94)	1.37(1.09,1.72)	0.005*
S7	rs1333049	0.94	1.21 (1.03,1.43)	1.40(1.05,1.87)	0.013*
S8	rs10757283	0.27	1.30 (1.05,1.62)	1.47(1.08,2.01)	0.01*
S9	rs1333047	0.10	0.973(0.878,1.078)	0.92 (0.67,1.25)	0.53
S10	10757277	0.51	1.063(0.875,1.293)	1.10 (0.81,1.49)	0.54
S11	10757279	0.32	1.026(0.841,1.252)	1.04 (0.76,1.41)	0.82
S12	rs3135506	0.09	1.26(1.01,1.57)	1.4 (1.02,1.91)	0.03*
S13	rs1558861	0.26	1.266(1.014,1.584)	1.44 (1.05,1.98)	0.02*
S14	rs662799	0.40	0.91(0.84,0.99)	0.60 (0.43,0.84)	0.03*
S15	rs10750097	0.19	1.50(1.22,1.84)	1.8(1.37,2.51)	0.0001*
S16	rs599839	0.06	1.19(1.04,1.37)	1.46(1.09,1.94)	0.009*
S17	rs646776	0.51	0.829(0.740,0.929)	1.62(1.21,2.17)	0.001*
S18	rs3846663	0.09	0.810(0.720,0.911)	1.72 (1.27-2.32)	0.0003*
S19	rs2271293	0.31	1.027(0.831,1.269)	1.039(0.764,1.413)	0.8

HWE = Hardy Weinberg equilibrium test (in control only), OR = odd ratio (representing increase risk of MI per risk allele, RR = relative risk, †= Fisher's *P* value

Table 4.7 Genotype frequencies of all variants associated with MI

SNP	SNP_ID	Genotype	No.(frequency)		Pearson's	Fisher's
			case	control	*P*	*P*
S1	rs4977574	AA	87 (0.458)	109(0.568)	0.09	0.09
		AG	82 (0.432)	67(0.349)		
		GG	21 (0.111)	16(0.083)		
S2	rs2891168	AA	60 (0.319)	85(0.467)	0.014	0.014*
		AG	102 (0.543)	78(0.429)		
		GG	26 (0.138)	19(0.104)		
S3	rs2383206	AA	80 (0.417)	106(0.552)	0.0288	0.0289*
		AG	95 (0.495)	72(0.375)		
		GG	17 (0.089)	14(0.073)		
S4	rs2383207	AA	80 (0.391)	107(0.557)	0.001	0.001*
		AG	84 (0.438)	69(0.359)		
		GG	33 (0.172)	16(0.083)		
S5	rs10811656	CC	82 (0.43)	111(0.58)	0.006	0.006*
		CT	85 (0.443)	65(0.34)		
		TT	25 (0.13)	14(0.07)		
S6	rs10757278	AA	81 (0.42)	113(0.59)	0.004	0.004*
		AG	94 (0.49)	65(0.34)		
		GG	17 (0.089)	14(0.03)		

Table 4.7 continue

SNP	SNP_ID	Genotype	No frequency		Pearson's *P*	Fisher's *P*
			Cases	Control		
S7	rs1333049	CC	44 (0.232)	34(0.177)	0.0664	0.0663
		CG	89 (0.468)	79(0.411)		
		GG	57 (0.300)	79(0.411)		
S8	rs107572783	CC	83 (0.449)	105(0.547)	0.05	0.05*
		CT	73 (0.395)	70(0.365)		
		TT	29(0.157)	17(0.089)		
S9	rs1333047	AA	82 (0.471)	93(0.484)	0.7	0.71
		AT	64 (0.368)	74(0.385)		
		TT	28 (0.161)	25(0.130)		
S10	rs10757277	AA	32 (0.176)	24(0.132)	0.44	0.455
		AG	70 (0.385)	78(0.429)		
		GG	80 (0.440)	80(0.44)		
S11	rs10757279	AA	20 (0.108)	24(0.132)	0.4	0.4
		AG	90 (0.484)	76(0.418)		
		GG	76 (0.409)	82(0.451)		
S12	rs3135506	CC	18 (0.094)	18(0.094)	0.014	0.015*
		CG	92 (0.479)	65(0.339)		
		GG	82 (0.427)	109(0.568)		

Table 4.7 continue

SNP	SNP_ID	Genotype	No frequency		Pearson's *P*	Fisher's *P*
			Cases	Control		
S13	rs1558861	CC	31 (0.168)	16(0.083)	0.04	0.04*
		CT	63 (0.341)	68(0.354)		
		TT	91 (0.49)	108(0.56)		
S14	rs662799	AA	96 (0.50)	120(0.62)	0.041	0.041*
		AG	84 (0.43)	61(0.31)		
		GG	12 (0.06)	11(0.05)		
S15	rs10750097	AA	37 (0.193)	18(0.094)	0.0006	0.0006*
		AG	84 (0.438)	69(0.36)		
		GG	71 (0.37)	105(0.54)		
S16	rs599839	AA	71 (0.37)	49(0.259)	0.04	0.04*
		AG	76 (0.396)	81(0.429)		
		GG	45 (0.23)	59(0.312)		
S17	rs646776	AA	68 (0.354)	88(0.458)	0.004	0.004*
		AG	77 (0.401)	81(0.422)		
		GG	47 (0.245)	23(0.120)		
S18	rs3846663	CC	75 (0.399)	98(0.510)	0.075	0.075
		CT	86 (0.457)	75(0.391)		
		TT	27 (0.144)	19(0.099)		

Table 4.7 continue

SNP	SNP_ID	Genotype	No frequency		Pearson's *P*	Fisher's *P*
			case	control		
S19	rs2271293	AA	27 (0.144)	21(0.109)	0.42	0.42
		AG	64 (0.342)	76(0.396)		
		GG	96 (0.513)	95(0.495)		

MI= myocardial infarction, SNP= Single nucleotide polymorphism

4.9 Genetic association of *APOA5* variants with triglycerides and demographic variables

Most replicated SNPs rs662799 and rs3135506 of *APOA5* were analyzed for association with plasma triglycerides and demographic variables Table 4.8. The triglyceride raising alleles G of rs662799 and allele C of rs3135506 were significantly associated with increased risk of MI. Mean plasma triglycerides level for variant rs662799 of homozygous GG (266 mg/dl) was higher in MI patients as compared to homozygous AA (189 mg/dl) patients. In MI subjects, the unadjusted geometric mean (β) of plasma triglyceride for SNP rs662799 was (1.183, 1.186 and 1.243) in subjects with AA, AG and GG genotypes, respectively. Consistent with these results, MI patients carrying the CC genotype for variant rs3135506 had higher triglyceride level (β = 1.22, 95% CI, 0.96-1.56) than CG (β = 1.18, 95% CI, 0.93-1.51) or GG (β = 1.18, 95% CI, 0.92-1.56) genotypes. Both variants (rs662799, rs3135506) of *APOA5* showed significant association with plasma triglyceride levels (*P*=0.03, *P*=0.05 respectively) among MI patients (Table 4.8).

The regression analysis of *APOA5* for other demographic variables such as age, sex, BMI, smoking, hypertension and diabetes was also performed. The analysis showed diabetes as a significant risk factor for the onset of MI (*P* = 0.04 and 0.02 respectively) in

comparison between genotypes of both variants (rs662799, rs3135506) (Table 4.9). The cases with genotype AA (51%) of rs662799 (*APOA5*) having diabetes are at high risk of MI. Genotype GG (44.9%) of rs3135506 was playing role in onset of MI in comparison to CG (40.8%) and CC (14.3%) genotypes among patients also having diabetes. While other independent risk factors did not revealed any association with *APOA5* genotypes and triglyceride levels and were similar to the main cohort. As shown in Table 4.9.

Table 4.8 *APOA5* (rs662799 and rs3135506) genotypes in relation to plasma level of triglycerides

	rs662799			**rs3135506**		
Parameters	**AA**	**AG**	**GG**	**CC**	**CG**	**GG**
% (cases/control)	50.0/62.5	43.8/31.8	6.3/5.7	18/9.3	65/33.7	109/56.5
Cases (mean ± SE) mg/dl	189± 3.3	192± 3.3	**266 ± 7.9**	**244.3 ± 3.8**	189 ± 3.2	191±3.7
Control (mean ± sd) mg/dl	126±2.3	122±2.8	123±4.8	125±4.9	121±2.5	127±2.6
β coefficient	1.183	1.186	1.243	1.22	1.18	1.18
95% CI	0.92-1.51	0.92-1.51	0.97-1.58	0.96-1.56	0.927-1.51	0.92-1.51
P†	0.21	0.2	**0.03**	**0.05**	0.21	0.2

P† was tested by multi- logistic linear regression of cases; β = beta, CI = confidence interval, bold letters = representing significant values.

Table 4.9 Comparison of demographic features between two alleles of rs662799 and rs3135506 for MI patient

parameters	**rs662799 AA (n=96)**	**rs662799 AG (n=84)**	**rs662799 GG(n=12)**	***P* value**
Age (year)	56.8±1.07	53.7±1.2	57±2.8	0.07
BMI (kg/m^2)	29.4±2.3	26.8±0.4	28.5±1.8	0.58
Smoking (%)	49.5	44.3	6.3	0.91
Life style (%)	49.8	43.5	6.7	0.74
Family history (%)	49.1	44.8	6.7	0.71
Hypertension (%)	43..5	48.1	8.3	0.08
Diabetes (%)	**51**	38.8	10.2	**0.04***
	rs3135506 CC (n=18)	**rs3135506 CG (n=92)**	**rs3135506GG (n=82)**	***P* value**
Age (year)	59.8±2.2	53.8±1.1	56.7±1.1	0.06
BMI (kg/m^2)	27.8±1.5	26.8±0.5	29.8±2.6	0.48
Smoking (%)	9.4	48.8	41.8	0.7
Life style (%)	10.84	47.5	41.7	0.16
Family history (%)	10.6	49.1	40.3	0.05
Hypertension (%)	10.2	50.9	38.9	0.48
Diabetes (%)	14.3	40.8	**44.9**	**0.02***

All continuous variables are expressed in means ± standard error (SE) and categorical variables are expressed in percentage, bold letter= higher values, *=P <0.05

4.10 Haplotypes associated with MI in the Pakistani (Punjab) population

Haplotype analysis was done for the 4 SNPs of 9p21 locus and 4 genetic variants of *APOA5* locus which were significantly associated with MI. It provided further support for the association of 9p21 locus and lipid metabolism related variants with MI.

4.10.1 Linkage disequilibrium (LD) and haplotype analysis of 9p21.3 locus

LD block map of associated 8 SNPs in 9p21.3 locus (*CDKN2A/B* gene) was shown in Figure 4.9a. (Annexures VI). In this Figure each diamond representing LD coefficient (D′) values, while red color of diamonds depicted strong association with disease. The D′ values of S1, S2, S7 and S8 indicated these SNPs were not in strong LD. Individual block map was also constructed in Figure 4.9b and 4.8c respectively representing MI patients with D' 99% (0.99) and correlation coefficient (r^2 = 0.94) respectively. The haplotype analysis for highly significant genetic variant S3, S4, S5 and S6 (chromosome 9p21.3) were shown in Table 4.10. Individual haplotypes of related SNPs (S3, S4, S5, and S6) with haplotype G-A-T-G frequency of 33% was significantly higher (*P* = 0.001) in MI group as compared to control 23%. In constrat A-G-C-A and G-G-C-A haplotypes were significantly lower in MI patients (60%, 3% respectively) verses control (70%, 4% respectively) (Table 4.10).

Table 4.10 Haplotype analysis of SNPs: S3, S4, S5 and S6

Haplotype	Cases (frequency %)	Control (frequency %)	Pearson's p
A- A- C- A	15.0(3.9)	8.00 (21)	0.144
A- A- T- G	0.00 (0.00)	6.23 (16)	0.01*
A- G- C- A	231.9 (60)	265.8 (70)	**0.006***
G- A- T- G	**126.9(33)**	**86.8 (23)**	**0.001***
G- G- C- A	1.00 (3)	13.23 (4)	0.0015*
A- A- T- A	8.02 (1.)	0.00(0.00)	0.0046*

Total cases=384, total control=380. Bold value representing risk haplotype
Global chi^2 is 36.746540 while df=5 (frequency < 0.01 in both control & case has been dropped.)
Fisher's P value is 3.77e-015; Pearson's P value is 6.97e-030

Pair-wise Linkage Disequilibrium (LD) analysis

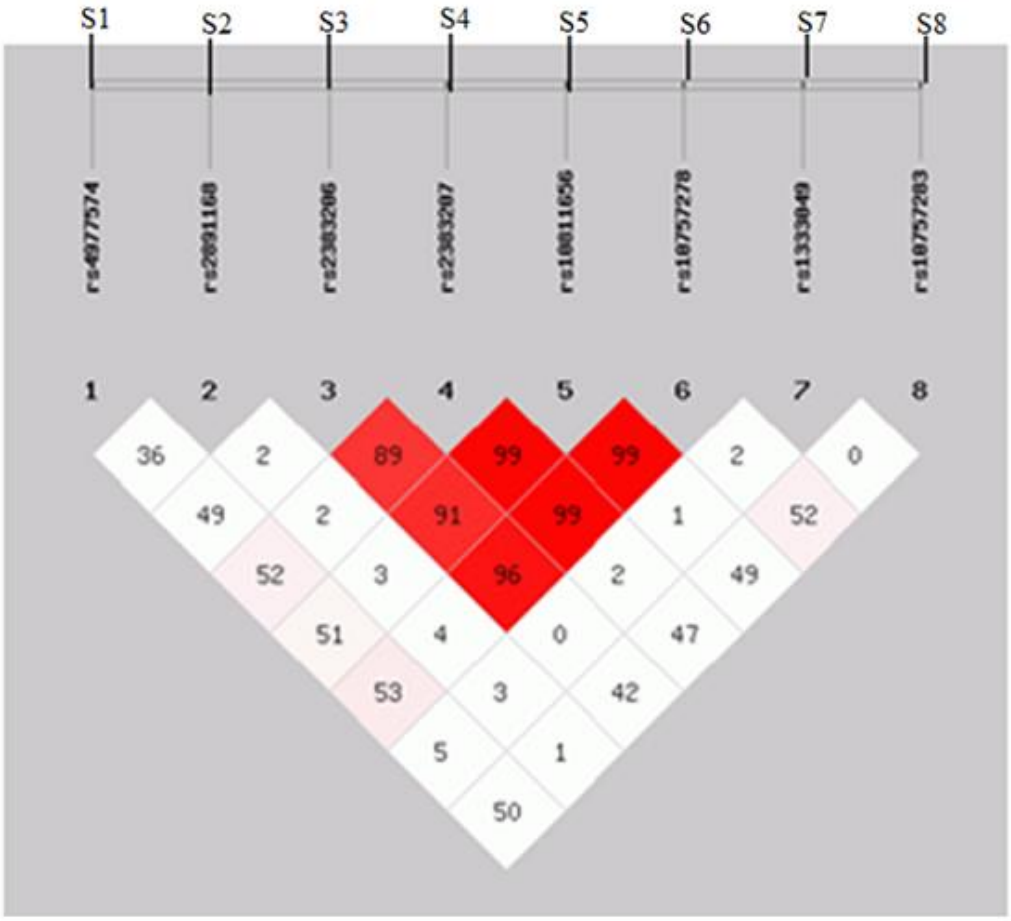


Figure 4.9 (a): LD block map of associated 8 SNPs in 9p21.3 locus. The pair-wise correlation between SNPs was shown as D' value in each diamond. Strong LD is evident with red colour (S3, S4, S5 and S6). The SNPs numbers are indicated at the top of block

Pair-wise Linkage Disequilibrium (LD) analysis

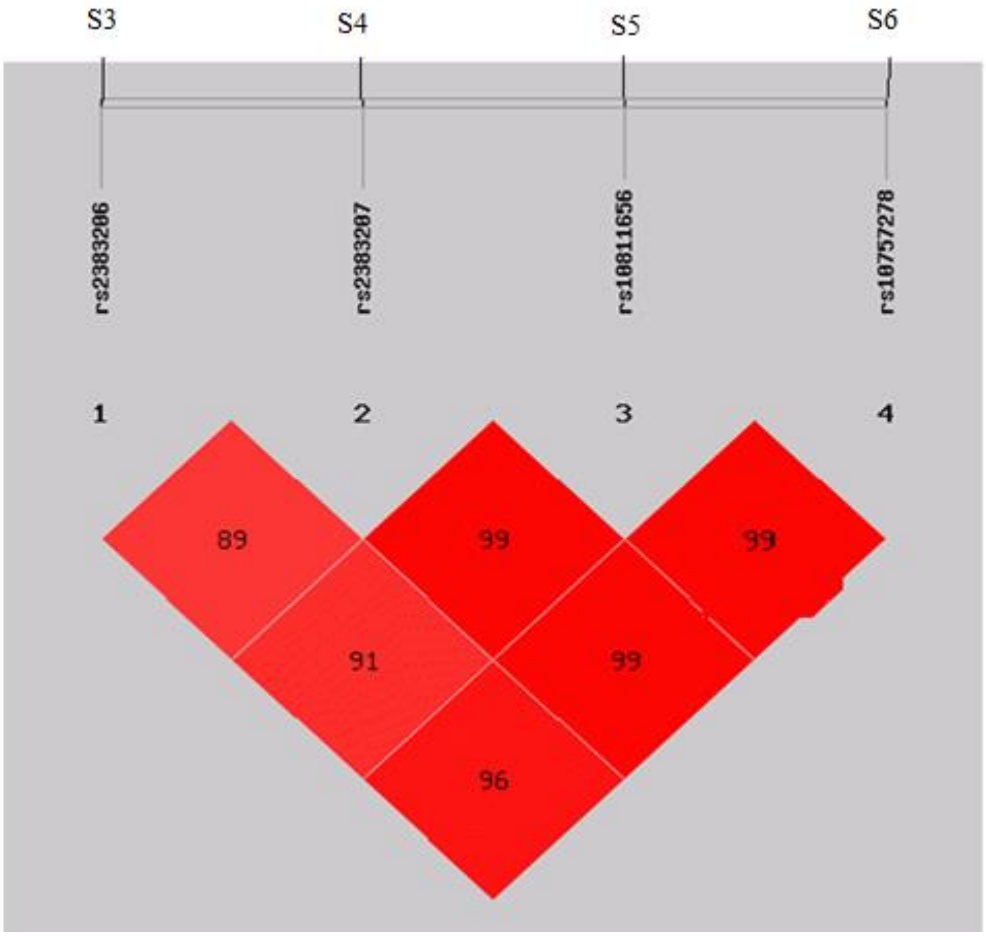


Figure 4.9 (b): Linkage disequilibrium (LD) block map for highly correlated 4 SNPs in 9p21.3 locus. It was measured as D' shown in each diamond. Strong LD is evident between all four SNPs (S3, S4, S5 and S6)

Pair-wise Linkage Disequilibrium (LD) analysis

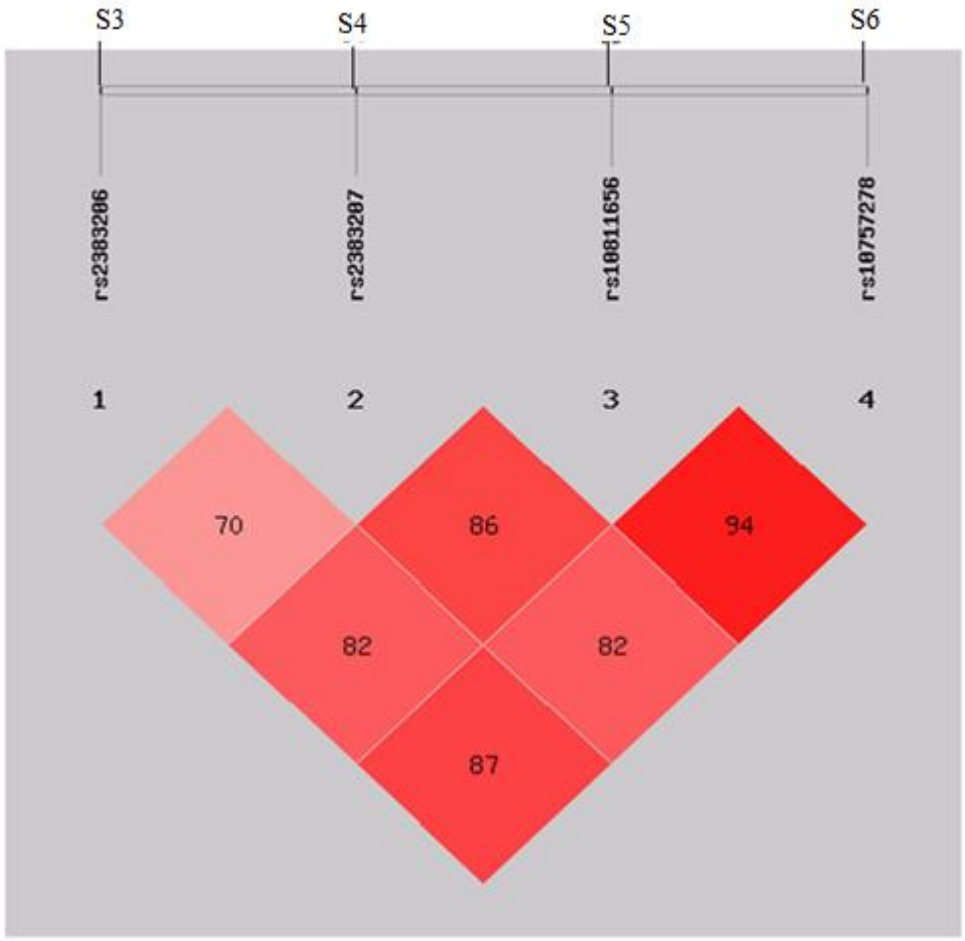


Figure 4.9 (c): LD block map for 4 SNPs in 9p21.3 locus, each diamond representing coefficient correlation (r^2) value. Dark red colour is representing strong association between SNPs.

4.10.2 LD and haplotype association analysis of *APOA5*

Other LD and haplotype analysis was done for the 4 SNPs (S12, S13, S14 and S15) residing in *APOA5* gene. It provided further support for the association of *APOA5* locus with MI. The haplotype results were shown in Table 4.11. The values of D′ and r^2 (Figure 4.10a and 4.10b) represented that rs1558861 (S13), rs662799 (S14) and rs10750097 (S15) were in strong LD (Annexures VI). The results for individual haplotypes of related SNPs (S12, S13, S14 and S15) confirmed that the frequency of C-T-G-A and G-C-A-G haplotypes were significantly higher ($P < 0.05$) in MI group as compared to control. In contrast G-T-A-G and C-C-G-A haplotypes were significantly lower ($P < 0.05$) in patients verses controls (Table 4.11).

Table 4.11 Haplotype analysis of SNP, rs3135506, rs1558861,rs662799, rs10750097

Haplotype	**Cases (freq %)**	**Control (freq %)**	**Pearson *P***	**Odd ratio[95%Cl]**
G-T-A-G	157.64(0.426)	261.88(0.682)	0.0001	0.357 [0.265~0.481]
C-C-G-A	36.38(0.098)	80.95(0.211)	0.0001	0.416 [0.273~0.634]
C-T-G-A	**63.42(0.171)**	2.05(0.005)	0.0001	39.334 [9.714~159.269]
G-C-A-A	15.10(0.041)	2.03(0.005)	0.000939	8.150 [1.870~35.521]
G-C-A-G	**56.14(0.152)**	9.09(0.024)	0.0001	7.534 [3.679~15.430]

Total control=384.0, total case=370.0; freq = frequency; bold values depicted risk haplotypes; Global chi^2 156.941238 while df=8 (frequency f < 0.01 in both case and control has been dropped); Fisher's *P* value is 3.77e-015; Pearson's P value is 6.97 e-030

Pair-wise Linkage Disequilibrium (LD) analysis

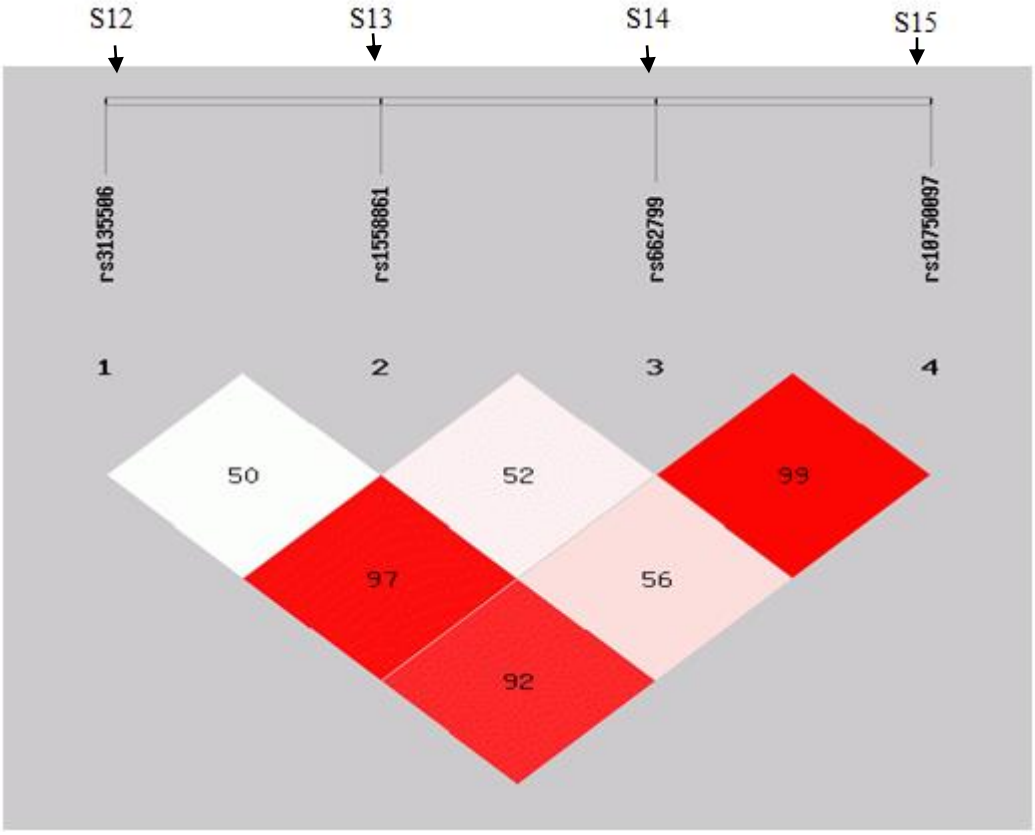


Figure 4.10(a): LD block map of 4 SNPs of *APOA5*. The pair-wise correlation between SNPs was measured as D' value in each diamond. White color is representing no association and red color showing strong association between SNPs. The SNPs numbers are indicated at the top of block map

Pair-wise Linkage disequilibrium (LD) analysis

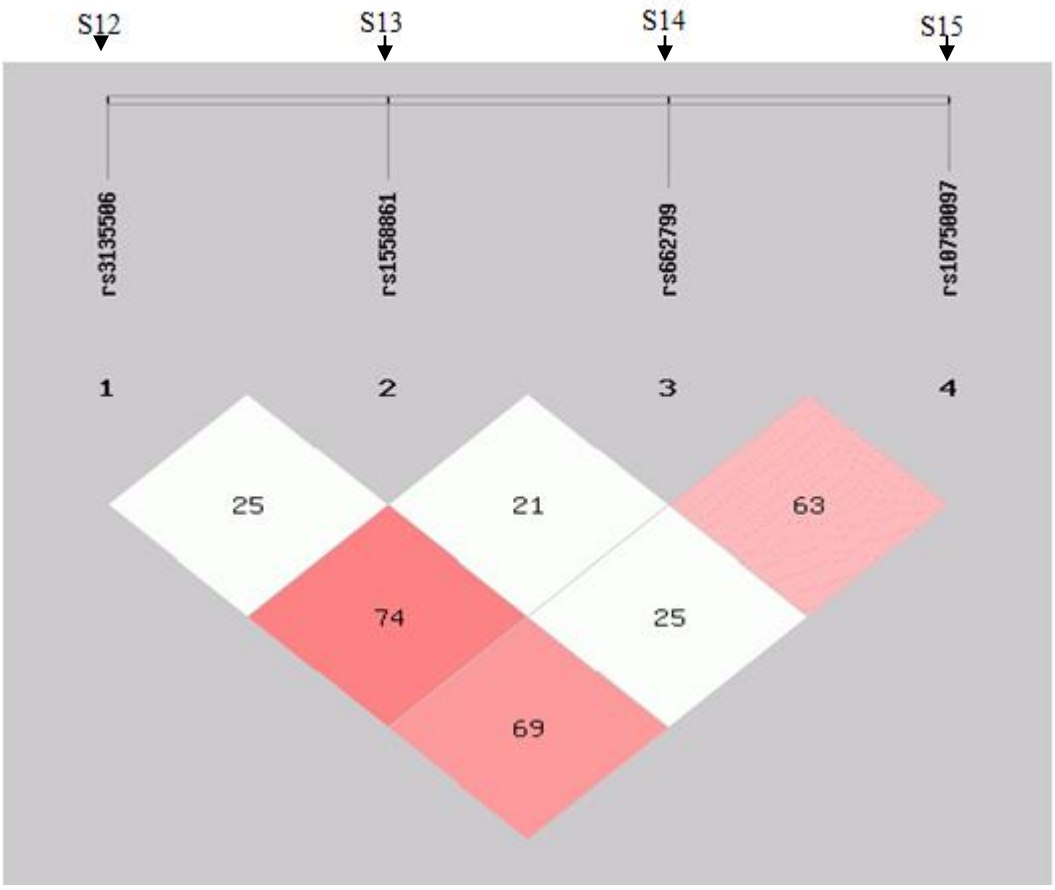


Figure 4.10 (b): LD block map of 4 SNPs of *APOA5*, each diamond representing r^2 values. Key as for figure 4.9 a

4.11 Family history association

4.11.1 Variants at 9p21.3 locus confer the risk of MI with positive family history

To determine the association between SNPs and positive family history of MI, patients with first degree relative who had suffered from MI (n = 121) and MI patients without family history (n = 71) were selected. Genotype distribution and allelic frequencies of significant SNPs were analyzed and compared with control group (n = 192). There were significant associations ($P < 0.05$) of 7 SNPs (S1, S2, S3, S5, S6, S7, and S8) with positive family history of MI as compared to control. The association of MI was not significant ($P > 0.05$) for the subgroup of MI without family history (Table 4.12). These results suggested the strong association of 9p21.3 locus with positive family history of MI.

Table 4.12 Association analysis of all SNPs with positive and negative family history of MI

	Negative FH (n = 71)		**Positive FH (n =121)**	
SNPs	***P***	**OR 95% Cl**	***P***	**OR 95% Cl**
rs4977574	0.19	1.31(0.86,2.01)	0.042*	1.43(1.01,2.04)
rs2891168	0.2	1.25(0.83,1.88)	0.004*	1.63(1.16,2.28)
rs2383206	0.15	1.36(0.89,2.06)	0.027*	1.48(1.04,2.10)
rs2383207	0.002	1.87(1.25,2.81)	0.0014	1.74(1.23,2.46)
rs10811656	0.06	1.47(0.96,2.25)	0.001*	1.79(1.26,2.54)
rs10757278	0.08	1.45(0.95,2.21)	0.006*	1.63(1.14,2.32)
rs1333049	0.49	1.14(0.77-1.69)	0.005*	1.58(1.14-2.19)
rs10757283	0.45	1.17(0.76,1.80)	0.003*	1.67(1.18,2.37)

P value all compared with192 normal controls, * = $P < 0.05$, FH = Family history, OR = Odd ratio, CI = Confidence interval, MI = myocardial infarction

CHAPTER 5

DISCUSSION

5.1 Environmental effect on MI

MI is multifactorial disease, caused by one or more genes in association and interaction with environmental factors (Van der Net *et al*., 2009). Risk factors associated with coronary heart disease in Pakistani population have been relatively less studied (Yusuf *et al*., 2001). The knowledge of these factors in different regions of Punjab, Pakistan is of great importance in order to develop national health strategies for their control. In demographic section of the current study, it had been found that cardiac disease and the risk factors: hypertension, diabetes and obesity were increasing in Pakistan. Current data depicted that male MI patients of rural and urban areas of Punjab were more susceptible at all age classes particularly at 41-60 years ($P < 0.015$) as compared to females. This is correlated with previous findings that middle aged (41- 60 years) men have 2 to 5 times higher risk of MI than women (Willcox *et al*., 2006; Reis *et al*., 2013) and women of young age are protected from risk of coronary heart disease (Toyofuku *et al*., 1996). Estrogen is thought to be a major contributor for premenopausal women's tendency to have normal blood pressure, higher levels of HDL-C, lower triglyceride levels and lower risk of MI than men (Wenger *et al*., 1985; Reslan and Khalil., 2012).

Current results indicated 56% patients with ST Segment Elevation Myocardial Infarction (STEMI) as compared to 44% Non ST Segment Elevation Myocardial Infarction (NSTEMI) which appeared as major public health problem in Pakistan. Badran *et al.* (2009) previously studied 253 cardiac patients in Egypt, out of which 48% patients had more incidence of STEMI as compared to 22% NSTEMI patients The author also reported that STEMI was major issue worldwide as compared to NSTEMI. In current study prevalence of STEMI was higher than Egyptian population.

Smoking was reported as major risk factor for the early onset of myocardial infarction accounting 80% increased risk of cardiovascular disease for smokers while 30% for passive smokers (Yusuf *et al*., 2001). Smoking deteriorates the HDL-C, raises the blood pressure and releases free radicals which cause injuries to heart muscles. Among non smokers, who inhaled smoke with passive exposure also greatly increase risk of coronary heart disease (Jousilahti *et al*., 1998). Furthermore, the follow up study conducted in Bangladeshi population demonstrated that smoking cessation resulted in 65% reduced risk of heart diseases (Wu *et al.*, 2013). In current study, the prevalence of smoking was more in MI patients 60.9% as compared to 39% non smokers. Furthermore, 32% smokers were found in rural areas as compared to 28% in urban areas. The study reported in rural and urban areas of West Bengal (Eastern part of India) also indicated 62.3% prevalence of smoking among urban cardiac patients and 16.6% smoking in rural cardiac patients which appeared as higher than Pakistani urban MI patients (Misra, 2000). The positive association between tobacco smoking and cardiac disease among both men and women was clearly demonstrated in both USA (U.S. Department of Health and Human Services, 2004) and Shanghai China (Yuan *et al*., 1996). The study conducted on Bangladeshi population indicated that tobacco smoking results in increase premature deaths due cancer and cardiac diseases among both men and women (Wu *et al.*, 2013). In addition a study reported in Thailand indicated a 2-3 times elevated death rate in smokers of all age group in comparison with non-smokers of related ages (Pratipanawatr *et al*., 2013).

Sedentary life style is also an important risk factor for MI (Jayalakshmi *et al*., 2011). In present study 70% of MI patients have sedentary life style where it was more 41% in urban areas as compared to 29% in rural areas. Previously 26% prevalence of sedentary life style was reported among coronary heart disease patients in Peshawar, Pakistan (Faisal *et al.*, 2011), indicating 44% higher prevalence of sedentary life style with MI patients in north Punjab Pakistan. Disturbance in metabolic pathways of important compounds (glucose imbalance, lipid metabolism) and heart functions were associated with physical inactivity in Australian and Canadian population (Healy *et al*., 2007; Katzmarzyk *et al*., 2009). In an animal modeling study using mice, it was

demonstrated that the physical exercise can improve functioning of left ventricle with reducing the risk of MI (de Waard and Duncker, 2009).

Hypertension is one of the important risk factor responsible for atherosclerotic events (Mc Gee, 2005). In present investigation, hypertension emerged as powerful risk factor among 37% MI Patients. However, 19.4% urban patients of MI have complaint of hypertension as compared to 17% in rural patients. Dietary habits of spicy food (high concentration of salt) and high prevalence of obesity with sedentary life style could participate in stimulating hypertension which in turn is greatly associated with MI (McGee, 2005). Many independent studies have reported higher prevalence of hypertension in Pakistan than other South Asian countries (Dodani *et al*., 2004; Iqbal *et al*., 2004; Javed *et al*., 2006). The high prevalence of hypertension may be due to poor economic conditions and lack of proper health care system in Pakistan.

Rees *et al*. (2011) demonstrated diabetes as major public health issue in Pakistan and is an important cause of cardiovascular disease (Fuuler *et al*., 2001; Jafar *et al*., 2004). The onset of diabetes in Asian population was reported at young age which varied according to culture, ethnicity and urbanization (Chen *et al*., 2009). In current study the distribution of type 2 diabetes was 19.4% in MI patients while it was more prevalent 13% among urban patients as compared to 7% in rural patients. The prevalence of diabetes has been reported to be significantly higher in urban population as compared to rural population of South India which is comparable with Pakistani population (Mohan and pradeepa, 2009). The difference in the prevalence of diabetes can be due to change in life style or environmental conditions in urban and rural areas of both countries (Ramachandran *et al*., 2008). Moreover, the higher prevalence of diabetes has been reported in cardiovascular patients in United State (Rosamond *et al*., 2008).

Hyperlipidemia (serum cholesterol level > 200mg/dl) is an important risk factor for the development of cardiac diseases (Yusuf *et al*., 2004). High lipid level can cause blockage of coronary arteries and increase the risk of heart attack. The distribution of hyperlipidemia was 26% among MI patients in Punjab Pakistan where susceptibility was more 19.4% in rural MI patients as compared to 7 % in urban patients. In India 22% of

rural population was with hypercholesterolemia (Gupta *et al*., 2003) which is slightly higher to Pakistani (Punjab) population. The higher and comparable cases of hyperlipidemia in Pakistani and Indian population can be attributed to the similar dietary habits and life style in both areas. Moreover it had been reported that over 7.3 million people in Pakistan have elevated cholesterol levels (Yusuf *et al*., 2004). The data obtained from Framingham heart study worldwide has demonstrated that morbidity and mortality due to coronary heart disease increases as blood cholesterol level rises (O'Donnell and Elosua, 2008).

The current study indicated a positive association of higher BMI and development of MI. However, positive family history of MI patients got heart disease even at lower BMI ≤ 25. In addition Ismail *et al*. (2004) reported that onset of heart disease at lower BMI (≤ 25) was due to consanguineous marriages in Pakistan. Nevertheless, significant ($P < 0.005$) high prevalence of overweight was found in urban areas (31.83%) as compared to rural areas (11.64%) of Pakistan. Gupta *et al.* (2007) reported that higher BMI was significantly ($P < 0.05$) associated with cardiovascular risk factors; hypertension, diabetes mellitus and lipid abnormalities resulting in the onset of cardiovascular disease. High prevalence of overweight among urban patients was may be due to less physical activity and unhealthy dietary practices which includes high consumption of saturated fats, refined carbohydrates as well as low consumption of fruits and vegetables.

Difference of disease treatment cost was insignificant ($P > 0.05$) among all economic classes studied presently. Mean estimated annual cost per patient was recorded as 9524.53 PKRs (96.96 US$). The calculated cost for cardiac disease treatment varies among different countries e.g., from US$ 9512 (Belgium) to 18293 US$ (Austria) per year (Tarride *et al.,* 2009). The low and middle income people were facing the socioeconomic burden to get their treatment costs. Moreover, the mean calculated cost per patient for heart surgery was 600 thousand (6108.19 US$) in Pakistan. One third of Pakistani people are living in poverty and cannot afford the increasing treatment burden of such costly disease (Iqbal *et al*., 2004). Hence, due to limited resources the heart surgery cost sometimes leads to change in financial capacity of the patients

which is making them hypertensive. Suhrcke *et al.* (2006) had reported cost greatly influences complication of coronary heart disease due to financial burden. In USA treatment cost was reported to be 32,975 US$/year/patient (Tarride *et al.*, 2009).

5.2 Genetic association of MI

Overall Current study was the first to identify three novel SNPs rs10757278, rs10811656 and rs10757283 on chromosome 9p21.3 using 11 genetic markers, against MI in central Punjabi population of Pakistan. These SNPs were found in two genes (*CDKN2A/B*) on chromosome 9p21.3 are involved in transcription of two proteins (p16 / p15) which are inhibitors of cell cycle and were found to be involved in formation of atherosclerotic plaque (Wei *et al*., 2013). The association we observed in Pakistani population could not be replicated in other South Asian populations however, the comparable mutations were already documented in many ethnicities such as Icelandic, Caucasians, South Korean, Chinese and German populations (Helgadottir *et al*., 2007; Kathiresan *et al.*, 2008; Shen *et al*., 2008; Zeng *et al*., 2013; Samani *et al*., 2007).

The rs4977574 from gene *CDKN2A/B* having minor allele G frequency greater in patients than control was found significantly associated ($P = 0.037$) with MI. It appeared that the allele G of same SNP was responsible for atherosclerosis risk development in Pakistani population. Our findings are in consistent with previous large scale studies on Caucasian, Lebanon and Hispanic populations (Kathiresan *et al.*, 2009; Saade *et al*., 2011; Qi *et al*., 2013) where risk allele G was significantly associated with increased risk of coronary heart disease. In addition a neighboring SNP rs2891168 located at the distance of 45 base pair downstream with risk allele G was significantly associated ($P < 0.05$) with the onset of MI. This is comparable with the two independent studies in Ontario, London and Italian populations which confirm the G as a risk allele for the development of coronary heart disease ($P < 0.05$) (Lanktree *et al*., 2008; Shen *et al*., 2008).

The SNPs rs2383206, rs2383207 and rs1333049 from gene *CDKN2A/B* were in strong association with MI in single site and genotype analysis ($P < 0.05$). Ahmad *et al*. (2013) strengthen the association of rs1333049 with risk allele C against MI in North

(Islamabad, Murree) of Pakistan. The same findings were found in Canadian and Icelandic studies for association with cardiovascular disease (Helgadottir *et al*., 2007; Mc Pherson *et al*., 2007). Moreover, the SNPs rs10757278 (risk allele G), rs10811656 (risk allele T) located only 5 base pair (bp) apart from each other were significantly related ($P < 0.05$) with disease. In consistent to current findings Zeng *et al*. (2013) has reported genetic marker rs10757278 at 9p21.3 locus (*CDKN2A/B*) was significantly associated with coronary heart disease in Chinese Han Population. Many independent studies of different races (Korea, Caucasian, and Iceland) have also depicted strong association of SNPs with cardiac disease (McPherson *et al*., 2007; Helgadottir *et al*., 2007; Shen *et al*., 2008).

The *ANRIL* promoter region have obligatory site for Zn-finger proteins that are important for transcription of *CDKN2A/B* (Rodriguez *et al.,* 2010). The genetic marker rs10757283 coupled with *ANRIL* gene at base position of 22134172 in chromosome 9p21.3 locus with risk allele T significantly contributed with the onset of MI at allele and genotype levels ($P < 0.05$). Current results contradict prior report for SNP rs10757283 association with coronary heart disease in Chinese population where risk allele C was associated with disease (Cheng *et al*., 2011). Moreover present findings support prior association of rs10757283 risk allele T with coronary heart disease in European population (Silander *et al*., 2009). The difference of risk allele between populations may be due to diversity in defining phenotypes.

In present study none of the 3 SNPs rs1333047, rs10757277 and rs10757279 (chromosome 9p21.3 locus) depicted significant association with MI. These findings are in contrast with prior report by Zeng *et al.* (2013) that these 3 SNPs (rs1333047, rs10757277 and rs10757279) were significantly associated with coronary heart disease in Chinese Han population. The negative results of these SNPs are useful to consider since the difference in study population and ethnicity due to environment, life style or mutations specific to populations.

5.2.2 Genetics of lipid metabolism related SNPs

The Apolipoprotein gene *APOA5* has been related previously with onset of various diseases such as thickness of common carotid artery intimae media and greatly increased in levels of triglycerides which leads to atherosclerosis, furthermore involved in atherosclerotic plaque composition (Garelnabi *et al*., 2013).

APOA5 gene was previously replicated from Chinese population in association with various cardiovascular diseases (Cheng *et al*., 2011). Currently the gene was replicated in Pakistani population and identified with four genetic markers (rs3135506, rs1558861, rs662799 and rs10750097) significantly associated ($P < 0.05$) with MI. The patients with minor allele C for SNPs rs3135506 and rs1558861 were at higher risk of MI onset. Minor allele (C) of SNP rs3135506 was also found to be involved in changing the signal activity of *APOA5* through amino acid replacement thus lowering the amount of functioning protein in British population (Talmud *et al*., 2004). The frequency of minor allele G for genetic markers rs662799 and rs10750097 from *APOA5* was higher in cases 28% and 41% in comparison to control 20% and 27% respectively. Minor allele G frequency (28%) of *APOA5* SNP rs662799 was comparable with Chinese population (29.9%) (Liu *et al*., 2009). However, the same allele was found in low frequency (0.8%) in European white population (Pennacchio *et al*., 2001). Present results are in contrast to previous findings where allele C was minor with 32% frequency among cardiovascular patients for SNP rs662799 in population of Morocco (Ouatou *et al*., 2014). These variations among gene frequency were may be due to ethnic diversity between populations.

Furthermore, the two genetic markers (rs662799 and rs3135506) from *APOA5* were found to be significantly associated with plasma triglyceride levels. Polymorphisms in database SNP rs662799 confined in the promoter region of *APOA5* plays role in *APOA5* mRNA translation, which in turn results in less *APOA5* levels and then it leads to higher plasma triglyceride levels (Palmen *et al*., 2008). The mean plasma triglyceride levels for variant rs662799 homozygous for G allele was 266 mg/dl higher than homozygous for allele A (189 mg/dl) in MI patients. In contrast to current findings mean

plasma triglyceride level for SNP rs662799 with genotype GG 112 mg/dl and genotype AA 90 mg/dl was reported in Australian population (Laurila *et al*., 2010). The higher levels of triglycerides in Pakistani population than Australian population may be due to the heterogeneity and life style including eating habits etc. Moreover genetic variant rs3135506 was significantly associated with mean plasma triglyceride level of 244 mg/dl for CC genotype in association with GG and CG with plasma triglyceride concentration of 191mg/dl and 189 mg/dl in MI patients. In contrast to present findings mean plasma triglyceride levels for SNP rs3135506 with genotype homozygous for C was 145 mg/dl, 90 mg/dl among homozygous G and 80 mg/dl for heterozygous was also lower in Australian population (Laurila *et al*., 2010). Furthermore, Ken-Dror *et al.* (2010) reported minor allele C of the same SNP was associated with 34% increased level of triglyceride concentration in immigrants to Israel**.** The results are also consistent with the previous findings that *APOA5* one of the most important genetic determinants affecting triglyceride levels which lead to atherosclerosis in German population (Cullen, 2000; Li *et al*., 2007).

There was no significant association among genotypes of the same SNPs (rs662799 and rs3135506) with demographic characteristics except diabetes. The diabetes was found significant (P = 0.04) risk factor among MI patients for homozygous AA in comparison with AG and GG genotypes of SNP rs662799. Diabetes was reported as strong predictor of cardiovascular disease in European population and in white population of USA (Qi *et al*., 2013; Adams *et al*., 2014).

Three genes (*PSRC1 /CELSR2 /SORT)* located on chromosome 1p13. SNP were located in the intergenic region of these three genes (Kooner *et al*., 2008) and taking part in regulating plasma low LDL-C levels, mutations in these genes can cause imbalance in LDL-C levels (Linsel-Nitschke *et al*., 2010). Genetic variants within this region, rs599839 and rs646776 risk allele G were significantly associated with MI ($P < 0.05$). Both of these SNPs were increasing the susceptibility of MI in Pakistani population. Previously it was reported same allele G associated with reduce risk of myocardial infarction in New Zealand (Muendlein *et al*., 2009). The contradictory results depicted that two SNPs (rs599839, rs646776) were reported protective against the development of

MI in New Zealand natives However, were at high risk in Pakistani population. Moreover, previously reported both variants (rs599839, rs646776) has been associated with increase levels of LDL-C which ultimately was associated with coronary atherosclerotic lesion in Europeans, South Asian and Hispanics (Kathiresan *et al*., 2009; Linsel-Nitschke *et al*., 2010; Saleheen *et al*., 2010; Qi *et al*., 2011).

In present study another genetic variant rs3846663 was identified in *HMGCR* gene, 3 hydroxy-3-methylglutaryl-coenzyme A-reductase (HMG-CoA reductase) playing key role in in cholesterol biosynthesis and elevating the risk of MI. The genotypes TT and CT were more susceptible in cases than control. The homozygous genotype TT of the SNP rs3846663 was also reported the risk allele associated with higher LDL-C level and high risk of CHD in Caucasians (Kathiresan *et al*., 2009).

The SNP rs2271293 (*NUTF2* gene) located on locus 16q22 controls HDL-C levels however, did not show any association with MI (P=0.8). Similar findings were reported by Voight *et al*. (2012) who found no association of variant rs2271293 (P = 0.10) in European population.

5.3 Linkage disequilibrium analysis

Linkage disequilibrium analysis was carried out between 4 variant (rs2383206, rs2383207, rs10811656 and rs10757278) of *CDKN2A/B* gene located in 9p21.3 locus and 4 genetic markers (rs3135506, rs1558861, rs662799 and rs10750097) of *APOA5* gene found on chromosome 11. Haplotypes were constructed, for those highly significant variants located in strong linkage disequilibrium.

Variants rs2383206, rs2383207, rs10811656 and rs10757278 (chromosome 9p21) were in strong LD. Current study identified G-A-T-G haplotype with frequency 33% in MI group was higher as compared to 23% in control. These findings support that certain haplotypes and LD patterns at 9p21.3 being useful in describing genotype disease association in MI. Haplotype and LD analysis of SNPs (rs3135506, rs1558861, rs662799 and rs10750097) residing in *APOA5* gene provided further support for the association of *APOA5* locus with MI. Present study identified C-T-G-A and G-C-A-G haplotypes as

risk haplotypes significantly (P = 0.0001) associated with MI. Laurila *et al.* (2010) identified *APOA5* risk haplotypes (C-A-C-G and G-G-C-G) were different from current study associated with lipid profile. This association of *APOA5* polymorphism (at genotyping, haplotype and linkage disequilibrium level) with MI, for the first time in our population, has been well established.

When the case group for 9p21.3 locus was divided into two subgroups; the significant association was identified only in sub group with positive family history of MI. This study is in line with findings of Shen *et al.* (2008) that strong family history was a strong predictor for onset of cardiac disease in Italian population.

5.4 Conclusion

Current data demonstrated people living in urban areas have higher level of MI risk factors (Physical inactivity, hypertension and diabetes) and therefore should be treated as "high risk group" for prevention and treatment. Moreover, this study demonstrated effective risk factors control is still poor in Punjab, Pakistan. Therefore public health programs including life style interventions along with different pharmacological therapy should be implanted in order to reduce the risk of coronary heart disease in Pakistan. Preventive efforts are needed to start early in life and should continue throughout the life course. Second phase of study (genetic analysis) is the first that has endeavored to explore the correlation between polymorphism of 9p21.3 locus and *APOA5* gene for the onset of MI in Pakistani population of province Punjab.

Present data indicates that the SNPs, rs10757278 risk allele G, the rs10811656 risk allele T and the rs10757283 risk allele T are associated with high risk of MI. Moreover, current findings support the role of *APOA5* in raising triglyceride level. The present confirms that Polymorphism in variants of *APOA5*, *CELSR2* and *HMGCR* genes may lead to the progression of MI. However, further studies are needed for genetic mapping to elaborate the genetic role of lipid metabolism related SNPs in Pakistan. Knowledge of genetic factors contributing to onset of MI may be helpful for diagnostic methods, treatment and preventive measures for this disease. There is a need for replicate

studies at large scale to determine the role of genetic variants in pathology of MI which can be helpful in early prediction as well as control and prevention of this disease.

CHAPTER 6

REFERENCES

Abduelkarem, A.R., El-Shareif, H.J and Sharif, S.I. 2012. Evaluation of risk factors in acute myocardial infarction patients admitted to the coronary care unit, Tripoli Medical Centre, Libya. *East. Mediterr. Health. J.* 1**8(4)**:332-6.

Abdullah, K.G., Li, L., Shen, G.Q., Hu, Y., Yang, Y., MacKinlay, K.G., Topol, E.J and Wang, Q.K. Four SNPS on chromosome 9p21 confer risk to premature, familial CAD and MI in an American Caucasian population (GeneQuest). 2008. *Ann. Hum. Genet*. **72**:654–657.

Adams, J.N., Raffield, L.M., Freedman, B.I., Langefeld, C.D., Ng, M.C., Carr, J.J., Cox, A.J and Bowden, D.W. 2014. Analysis of common and coding variants with cardiovascular disease in the diabetes heart study. *Cardiovasc. Diabetol*. **13**: 77.

Adler, A.I., Stratton, I.M., Neil, H.A.W., Yudkin, J.S., Matthews, D.R., Cull, C.A., wright, A.D., Turner, R.C and Holman, R.R. 2000. Association of systolic blood pressure with macrovascular and microvascular complications of type 2 diabetes (UKPDS 36): prospective observational study. *B.M.J.* **321**:412-419.

Ahmad, I and Shafique, Q. 2003. Myocardial infarction underage 40: Risk factors and coronary arteriographic findings. *Ann. King. Edward. Med. Coll*. **9(4)**:262-5.

Ahmad, W., Ali, I.S., Younas, A., Sadeque, A., Niazi, A.K., Niazi, S.H., Ali, S.H.B and Azam, M. 2013. Association of *ANRIL* polymorphism (rs1333049:C>G) with myocardial infarction and its pharmacogenomic role in hypercholesterolemia. *Gene*. **515:** 416-420.

Alam, A. Y., Iqbal, A., Mohamud, K. B., Laporte, R. E., Ahmed, A and Nishtar, S. 2008. Investigating socio-economic-demographic determinants of tobacco use in Rawalpindi, Pakistan. *B.M.C.Public. Health.* **8:**50.

Alberts, B., Johnson, A and Lewis, J. 2002. Transport into the Cell from the Plasma Membrane: Endocytosis. Molecular Biology of the Cell 4th edition New York: Garland Science.

Alfredo, M.J., Martínez, H.A., Enríquez, L., Moreno,A.M., Moreno, M.M and Martí A. 2007. Genetics of obesity. *Public. Health. Nutr*. **10 (10A)**:1138-44.

Afridi, A.K. and Khan, A. 2004. Prevalence and etiology of obesity-an overview. *Pak. J. Nutr*. **3**: 14-25.

Alnemer, K.A., AlFaleh, H.F and Butt, M. 2012. Impact of diabetes on hospital adverse cardiovascular outcomes in acute coronary syndrome patients: Data from the Saudi project of acute coronary events. *Saudi. Heart. Assoc*. **24(4)**: 225–231.

Alp, E., Menevse, S., Tulmac, M., Kan, D., Yalcin, R., Erkan, A.F and Cengel, A. 2009. Lack of association between matrix metalloproteinase-9 and endothelial nitric oxide synthase gene polymorphisms and coronary artery disease in Turkish population. *DNA. Cell. Biol*. **28(7)**:343-50.

Alpert, J.S., Thygesen, K., Antman, E and Bassand, J.P. 2000. Myocardial infarction redefined a consensus document of The Joint European Society of Cardiology/American College of Cardiology Committee for the redefinition of myocardial infarction. *J. Am. Coll. Cardiol*. **36(3)**: 959-69.

Ambrose, J.A and Barua, R.S. 2004. The pathophysiology of cigarette smoking and cardiovascular disease: an update. *J. Am. Coll. Cardiol.* **43(10)**:1731.

Anjos, L.A., Teixeira Fda, C., Wahrlich, V., Vasconcellos, M.T and Going, S. B. 2013. Body fat percentage and body mass index in a probability sample of an adult urban population in Brazil. *Cad. Saude. Publica*. **29(1):** 73-81.

Aziz, K.U., Faruqui, A., Manolio, T and Davis, C.E. 2005. Blood Pressure and Hypertension Distribution in a lower middle class urban community in Pakistan. *J.P.M.A.* **55**: 333.

Badran, H.M., Elnoamany, M.F., Khalil, T.S and Eldin, M.M. 2009. Age-related alteration of risk profile, inflammatory response, and angiographic findings in patients with acute coronary syndrome. *Clin. Med. Cardiol*. **3**:15-28.

Ballantyne, C.M., Olsson, A.G., Cook, T.J., Mercuri, M.F., Pedersen, T.R and Kjekshus, J. 2001. Influence of low high-density lipoprotein cholesterol and elevated triglyceride on coronary heart disease events and response to simvastatin therapy in 4S. *Circulation.* **104**: 3046–3051.

Basit, A and Shera, A.S. 2008. Prevalence of metabolic syndrome in Pakistan. *Metab. Syndr. Relat. Disord.* **6(3)**:171-5.

Battinelli, T. 2007. Physique, Fitness, and Performance, Second Edition. Nutrition and Heart disease, Taylor and Francis group. 151pp**.**

Bennett, D.A., Xu, P., Clarke, R., Zondervan, K., Palmer, A., Cardon, L., Peto, R., Lathrop, M and Rory. 2008. The exon 1-8C/G SNP in PSMA6 gene contributes only a small amont to the burden of myocardial infarction in 6946 cases and 2720 controls from a United Kindon population. *Eur. J. Hum. Genet*. **16:** 480-486.

Betticher, D.C., Thatcher, N., Altermatt, H.J., Hoban, P., Ryder, W.D and Heighway, J. 1995. Alternate splicing produces a novel cyclin D1 transcript. *Oncogene.* **11**:1005–11.

Bhalodkar, N.C., Blum, S., Rana, T., Bhalodkar, A., Kitchappa, R., Kim, K.S and Enas, E. 2004. Comparison of levels of large and small high-density lipoprotein cholesterol in Asian Indian men compared with Caucasian men in the Framingham Offspring Study. *Am. J. Cardiol.* **94**: 1561–1563.

Bhatnagar, D., Soran, H and Durrington, P.N. 2008. "Hypercholesterolaemia and its management". *B.M.J.* **337**: a993.

Bhopal R., Unwin N., White M., Yallop J., Alberti K. G. M. M.,Harland J., Patel S., Ahmad N., Turner C., Watson B., Kaur D., Kulkarni A., Laker M and Tavridou A.1999. Heterogeneity of coronary heart disease risk factors in Indian, Pakistani, Bangladeshi, and European origin populations: cross sectional study. *B.M.J.* **319**: 215–220.

Bogousslavsky, J. 2001. Drug Therapy for Stroke Prevention. Taylor and Francis Inc. London. Pp191.

Bostom, A.G., Cupples, L.A., Jenner, J.L., Ordovas, J.M., Seman, L.J., Wilson, P.W., Schaefer, E.J and Castelli, W.P. 1996. Elevated plasma lipoprotein and coronary heart disease in men aged 55 years and younger. A prospective study. *J.A.M.A.* **276**:544–548.

Bray, G.A and Bouchard, C. 2003. Handbook of Obesity: Clinical Applications. CRC Press. Amazon.Co 42 pp.

Broadbent, H.M., Peden, J.F., Lorkowski, S., Goel, A and Ongen, H. 2008. Susceptibility to coronary artery disease and diabetes is encoded by distinct, tightly linked SNPs in the ANRIL locus on chromosome 9p. *Hum. Mol. Genet.* **17**:806–814.

Brundtland, G.H. 2002. World Health Organization. World Health Report 2002 – Reducing Risks: Promoting Healthy Lifestyles.Geneva, Switzerland

Burton, P. R., Clayton, D. G., Cardon, L. R., Craddock, N., Deloukas, P., Duncanson, A., Kwiatkowski, D. P, McCarthy, M. I., Ouwehand, W. H., Samani, N. J., Todd, J. A., Donnelly, P., Barrett JC, Burton PR, Davison D, Donnelly P, Easton D, Evans D, Leung, H. T., Marchini, J. L., Morris, A. P., Spencer, C. C., Tobin, M. D., Cardon, L. R., Clayton, D. G., Attwood, A. P., Boorman, J. P., Cant, B., Everson, U., Hussey, J. M., Jolley, J. D, Knight, A. S., Koch, K., Meech, E., Nutland, S., Prowse, C. V, Stevens, H. E., Taylor, N. C., Walters, G. R., Walker, N. M.,

Watkins, N. A., Winzer, T., Todd, J. A., Ouwehand, W. H., Jones, R. W., Mc-Ardle, W. L., Ring, S. M., Strachan, D. P., Pembrey, M., Breen, G., St-Clair, D., Caesar, S., Gordon-Smith, K., Jones, L., Fraser, C., Green, E. K., Grozeva, D., Hamshere, M. L., Holmans, P. A., Jones, I. R., Kirov, G., Moskvina, V., Nikolov, I., O'Donovan, M. C., Owen, M. J., Craddock, N., Collier, D. A., Elkin, A., Farmer., A., Williamson, R., McGuffin, P and Young, A. H., Ferrier. 2007. IN Wellcome Trust Case-Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature*. **447**: 661– 678.

Bush, W. S and Moore, J. H. 2012. Genome-wide association studies. *PLOS. Comput. Biol*. **8 (12)**: e1002822.

Cade, W.T. 2008. Diabetes-related microvascular and macrovascular diseases in the physical therapy setting. *Phys. Ther*. **88***:*1322–1335.

Capewell, S., MacIntyre, K., Stewart, S., Chalmers, J. W., Boyd, J., Finlayson, A., Redpath, A., Pell, J. P and Mc Murray, J. J. 2001. Age, sex, and social trends in out-of-hospital cardiac deaths in Scotland 1986-95: a retrospective cohort study. *Lancet*. **358**:1213-7.

Carantoni, M., Abbasi, F., Chu, L., Chen, Y.D.I., Reaven, G.M., Taso, P.S., Varasteh, B and Cooke, J.P. 1997. Adherence of mononuclear cells to endothelium in vitro is increased in patients with NIDDM. *Diabetes. Care*. **20:**1462-1465.

Cardon, L.R and Palmer, L.J. 2003. Population stratification and spurious allelic association. *Lancet.* **361**:598-604.

Chadha, S. L., Radharrishanan, S., Ramamchandran, K., Kaul, U and Gopinath, N. 1990. Epidemiological study of coronary heart disease in urban population of Delhi. *Indian. J. Med. Res*. **92**: 424-30.

Chaudhry, M.A., Waseem, M., Ahmad, F., Ashraf, M.Z and Bhatti, A. 2012. Frequency of Coronary Heart Disease: Risk Factors among Doctors of CMH Lahore Medical and Dental College, Lahore – Pakistan. *A.P.M.C*. **6**:2.

Chen, Y.C., Chen, Y.D., Li, X., Post, W., Herrington, D., Polak, J.F., Rotter, J.I and Taylor, K.D. 2009. The HMG-CoA reductase gene and lipid and lipoprotein levels: the multi-ethnic study of atherosclerosis. *Lipids*. **44(8)**:733-43.

Cheng, X.X., Shi, L., Nie, S., Wang, F., Li, X., Xu, C., Wang, P., Yang, B., Qingxian, Li. Q., Pan, Z., Li, Y., Xia, H., Zheng, C., Ke, Y., Wu, Y., Tang, T., Yan, X., Yang, Y., Xia, N., Yao, R., Wang, B., Ma, X., Zeng, Q., Tu, X., Liao, Y and Wang, Q. K. 2011. The Same Chromosome 9p21.3 Locus Is Associated With Type 2 Diabetes and Coronary Artery Disease in a Chinese Han Population. *Diabetes*.**60 (2)**: 680-684.

Chow, C.K., Islam, S., Bautista, L., Rumboldt, Z., Yusufali, A., Xie, C., Anand, S.S., Engert, J.C., Rangarajan, S and Yusuf, S. 2011. Parental history and myocardial infarction risk across the world: the INTERHEART Study. *J. Am. Coll. Cardiol*. **57**: 619–627.

Christenson, E and Christenson, R.H. 2013. The Role of Cardiac Biomarkers in the Diagnosis and Management of Patients Presenting with Suspected Acute Coronary Syndrome. *Ann. Lab. Med.* **33(5)**: 309–318.

Christen, W.G., Ajani, U.A., Glynn, R.J and Hennekens, C.H. 2000. Blood levels of homocysteine and increased risks of cardiovascular disease: causal or casual. *Arch Intern Med.* **160(4)**:422-434.

Critchley, J., Liu, J., Zhao, D., Wei, W and Capewell, S. 2004. Explaining the increase in coronary heart disease mortality in Beijing between 1984 and 1999. *Circulation*. **110(10)**:1236– 44.

Cullen, P. 2000. Evidence that triglycerides are an independent coronary heart disease risk factor. *Am .J. Cardiol.* **86**: 943–949.

Daly, A.K and Day, C.P. 2001. Candidate gene case-control association studies: advantages and potential pitfalls. *Br. J. Clin. Pharmacol*. **52:** 489–499.

Danik, J.S., Rifai, N., Buring.J. E.and Ridker, P. M. 2008. Lipoprotein (a), hormone replacement therapy, and risk of future cardiovascular events. *J. Am. Coll. Cardiol*. **52(2)**:124-131.

Daubert, M.A and, Jeremias, A. 2010. The utility of troponin measurement to detect moycardial infarction: review of the current findings. *Vasc. Health. Risk. Manag*. **6:**691–699.

De Caterina, R., Talmud, P.J., Merlini, P.A., Foco, L., Pastorino, R., Altshuler, D., Mauri, F., Peyvandi, F., Lina, D., Kathiresan, S., Bernardinelli, L., Ardissino, D and Aterosclerosi, G.I. 2011. Strong association of the APOA5-1131T>C gene variant and early-onset acute myocardial infarction. *Atherosclerosis.* **214(2)**:397-403.

De Waard, M.C and Duncker, D.J. 2009. Prior exercise improves survival, infarct healing, and left ventricular function after myocardial infarction. *J. Appl. Physiol.* **107:** 928–936.

Dehghan, A., van Hoek, M., Sijbrands, E.J., Oostra, B.A., Hofman, A., van Duijn, C.M and Witteman, J.C. 2008. Lack of association of two common polymorphisms on 9p21 with risk of coronary heart disease and myocardial infarction; results from a prospective cohort study. *B.M.C. Med*. **6**:30.

Di Angelantonio, E., Sarwar, N and Perry, P. 2009. Major lipids, apolipoproteins, and risk of vascular disease. *J. A.M.A.* **302**: 1993–2000.

Diaz, V.A., Mainous, A.G., Baker, R., Carnemolla, M and Majeed, A. 2007. How does ethnicity affect the association between obesity and diabetes? *Diabet. Med.* **24**: 1199-204.

Dinc, G., Gercekliogluu, G., Sozmen, K., Arık, H and Unal, B. 2013. Decreasing trends in cardiovascular mortality in Turkey between 1995 and 2008. *BMC. Public. Health.* **13:** 896.

Dodani, S., Mistry, R., Farooqi, M., Khwaja, A., Qureshi, R and Kazmi, K. 2004. Prevalence and awareness of risk factors and behaviors of coronary heart disease in an urban population of Karachi, the largest city of Pakistan: a community survey. *J. Public. Health.* **26:** 245–249.

Donner, M.G., Klein, G.K., Mathes, P.B., Schwandt, P and Richter, W.O. 1998. Plasma total homocysteine levels in patients with early-onset coronary heart disease and a low cardiovascular risk profile. *Metabolism.* **47***:*273–9.

Drazen, J.M., Yandava, C.N and Dube, L. 1999. Pharmacogenetic association between ALOX5 promoter genotype and the response to anti-asthma treatment. *Nat. Genet.* **22**:168–170.

Dunn, N.R., Arscott, A and Thorogood, M. 2001. The relationship between use of oral contraceptives and myocardial infarction in young women with fatal outcome, compared to those who survive: results from the MICA case-control study. *Contraception*. **63(2)**: 65-9.

Dunstan, D.W., Zimmet, P.Z., Welborn, T.A., De Courten, M.P., Cameron, A.J., Sicree, R.A., Dwyer, T., Colagiuri, S., Jolley, D., Knuiman, M., Atkins, R and Shaw, J.E. 2002. The rising prevalence of diabetes and impaired glucose tolerance: the Australian Diabetes. *Obesity and Lifestyle Study. Diabetes Care.* **25**: 829-834.

Eckel, R.H. 1997. A Statement for Healthcare Professionals from the Nutrition Committee, American Heart Association. *Circulation.* **96:** 3248-3250.

Edelstein, S and Sharlin, J. 2008. Life Cycle Nutrition: an Evidence-Based Approach. Jones & Bartlett .11pp 368.

Enas, E.A and Mehta, J. 1995. Malignant coronary artery disease in young Asian Indians: thoughts on pathogenesis, prevention, and therapy. Coronary Artery Disease in Asian Indians (CADI) Study. *Clin. Cardiol.* **18**: 131-5.

Enas, E.A and Senthilkumar, A. 2001. Coronary artery disease in Asian Indians. *J. Cardiol.* **1(2)**.

Eyre, H., Kahn, R and Robertson, R.M. 2004. Preventing cancer, cardiovascular disease, and diabetes: a common agenda for the American Cancer Society, the American Diabetes Association and the American Heart Association. *Circulation.* **109**: 3244-3255.

Ezzati, M. Hoorn, S.V., Rodgers, A., Lopez, A.D., Mathers, C.D and Murrary, C.J. 2003. Estimates of global and regional health gains from reducing multiple major risk factors. *Lancet*. **362**: 271–80.

Faisal, A.W., Ayub, M., Waseem, T., Khan, R.S and Hasnain SS. 2011. Risk factors in young patients of acute myocardial infarction. *J. Ayub. Med. Coll. Abbottabad.* **23(3)**:10-3.

Fang, J., Shaw, K.M and Keenan, N.L. 2011. Prevalence of Coronary Heart Disease --- United States, 2006—2010. *Morbidity and Mortality Weekly Report (MMWR).* **60(40):**1377-1381.

Ford, E.S and Jones, D.H. 1991. Cardiovascular health knowledge in the United States: findings from the National Health Interview Survey, 1985. *Prev. Med.* **20(6)**: 725-736.

France, M.W., Kwok, S., McElduff, P and Seneviratne, C.J. 2003. Ethnic trends in lipid tests in general practice. *Q. J. M.* **96 (12)**: 929-923.

Fuster, V and Kelly, B.B. 2010. Promoting Cardiovascular Health in the Developing World: A Critical Challenge to Achieve Global Health. Washington, DC*:* National Academic Press. ISBN-13: 978-0-309-14774-3.

Fuuler, J.H., Stevens, L.K and Wang, S.L. 2001. Risk factors for cardiovascular mortality and morbidity: the WHO Multinational Study of Vascular Disease in diabetes. *Diabetologia.* **44**:54-64.

Gabriel, R., Alonso, M., Segura, A., Tormo, M.J., Artigao, L.M., Banegas, J.R., Brotons, C., Elosua, R., Fernandez-Cruz, A., Muniz, J., Reviriego, B and Rigo, F. 2008. Prevalence, geographic distribution and geographic variability of major

cardiovascular risk factors in Spain. Pooled analysis of data from population-based epidemiological studies: the ERICE Study. *Rev. Esp. Cardiol.*. **61(10):** 1030-1040.

Gabriel, S.B., Schaffner, S.F., Nguyen, H., Moore, J.M., Roy, J., Blumenstiel, B., Higgins, J., Defelice, M., Lochner, A., Faggart, M., Liu-Cordero, S.N., Rotimi, C., Adeyemo, A., Cooper, R., Ward, R., Lander, E.S., Daly, M.J and Altshuler, D. 2002. The structure of haplotype blocks in the human genome. *Science*. **296**: 2225–2229.

Gale, E.A and Gillespie, K.M. 2001. Diabetes and gender. *Diabetologia*. **44(1)**: 3-15.

Gallagher, P.M., Meleady, R., Shields, D.C., Tan, K.S., McMaster, D., Rozen, R., Evans, A., Graham, I.M and Whitehead, A.S. 1996. Homocysteine and risk of premature coronary heart disease. Evidence for a common gene mutation, *Circulation.* **94**: 2154–2158.

Gao, F., Ihn, H. E., Medina, M. W and Krauss, R.M. 2013. A common polymorphism in the LDL receptor gene has multiple effects on LDL receptor function. *Hum. Mol. Genet*.**22:**1424–1431.

Garcia, C.K., Wilund, K., Arca, M., Zuliani, G., Fellin, R., Maioli, M., Calandra, S., Bertolini, S., Cossu, F., Grishin, N. Barnes, R., Cohen, J.S and Hobbs, H.H. 2001 Autosomal recessive hypercholesterolemia caused by mutations in a putative LDL receptor adaptor protein. *Science*. **292**: 1394–1398.

Garelnabi, M., Lor, K., Jin, J., Chai, F and Santanam, N. 2013. The paradox of ApoA5 modulation of triglycerides: evidence from clinical and basic research. *Clin. Biochem*. **46**: 12–19.

Gaziano, T.A., Bitton, A., Anand, S., Gessel, S.A and Murphy, A. 2010. Growing Epidemic of Coronary Heart Disease in Low- and Middle-Income Countries, *Curr*. P*robl. Cardiol*. **35(2):** 72-115.

Gholapa, N., Daviesb, M., Patel, K., Sattard, N and Khuntie, K. 2011. Type 2 diabetes and cardiovascular disease in South Asians. *Prime. Care. Diab*. **5**: 45–56.

Go, A.S., Mozaffarian, D., Roger, V.L., Benjamin, F.J.,Berry, J.D., Borden, W.B., Bravata, D.M., Dai, S., Ford, E.S., Fox, C.S., Franco, S., Fullerton, H.J., Gillespie, C., Hailpern, S.M., Heit, J.A., Howard, V.J., Huffman, M.D., Kissela, B.M., Kittner, S.J., Lackland, D.T., Lichtman, J.H., Lisabeth, L.D.,Magid, D., Marcus, G.M., Marelli, A., Matchar,D.V., McGuire, D.K., Mohler, E.R., Moy, C.S., Mussolino, M.E., Nichol, G., Paynter, N.P., Schreiner, P.J., Sorlie, P.D., Stein,J., Turan, T.N., Virani, S.S. Woo, D and Turner, M.B. 2013. Heart disease and stroke statistics--2013 update: a report from the American Heart Association. *Circulation*. **127**:e6-e245.

Goldstein, M.G and Niaura, R. 2000. Methods to enhance smoking cessation after myocardial infarction. *Med. Clinics. North. Am.* **84(1)**: 63 80.

Gupta, M., Singh, N and Verma, S. 2006. South Asians and cardiovascular risk: what clinicians should know. *Circulation*. **113(25)**:e924-9.

Gupta, R., Joshi, P., Mohan, V., Reddy, K.S and Yusuf, S. 2008. Epidemiology and causation of coronary heart disease and stroke in India. *Heart*. **94**:16-26.

Gupta, R., Gupta, V.P., Sarna, M., Prakash, H., Rastogi, S and Gupta, K.D. 2003. Serial epidemiological surveys in an urban Indian population demonstrate increasing coronary risk factors among the lower socioeconomic strata. *J. Assoc. Physicians. India*. **51**:470-7.

Hafizullah, M., Fawad, A., Saqib, M., Gul, A.M and Jan, H. 2010. Frequency of cardiovascular risk factors among prisoners. *Pak. Heart. J*. **43(1)**.

Hassan, M., Awan, Z.A., Gul, A.M., Shibzada, W.A and Hafizullah, M. 2005. Prevalence of Coronary Artery Disease in rural areas of Peshawar, *J.PM.I.* **19 (1)**: 14-22.

Healy, G. N., Dunstan, D.W., Salmon, J., Cerin, E., Shaw, J. E., Zimmet, P.Z and Owen N. 2007. Objectively measured light-intensity physical activity is independently associated with 2-h plasma glucose. *Diabetes. Care.* **30(6)**:1384–9.

Hebert, J.R and Kabat, G.C. 1990. Difference in dietary intake associated with smoking status. *Eur. J. Clin. Nutr*. **44**:185–193.

Helgadottir, A., Manolescu, A., Helgason, A., Thorleifsson, G., Thorsteinsdottir, U., Thorsteinsdottir1, U., Gudbjartsson, D.F., Gretarsdottir, S., Magnusson, K.P., Gudmundsson, G., Hicks, A., Jonsson, T., Grant, S.F.A., Sainz, J., O'Brien, S.J., Sveinbjornsdottir, S., Valdimarsson, E.M., Matthiasson, S.E., Levey, A.I., Abramson, J.L., Reilly, M.P., Vaccarino,V., Wolfe, M.L., Gudnason, V., Quyyumi, A.A., Topol, E.J., Rader, D.J., Thorgeirsson, G., Gulcher, J.R., Hakonarson, H., Kong, A and Stefansson, k. 2006. A variant of the gene encoding leukotriene A4 hydrolase confers ethnicity-specific risk of myocardial infarction. *Nat. Genet.* **38**:68–74.

Helgadottir, A., Thorleifsson, G., Manolescu, A., Gretarsdottir, S., Blondal, T., Jonasdottir, A., Jonasdottir, A., Sigurdsson, A., Baker, A., Palsson, A., Masson, G., Gudbjartsson, D.F, Magnusson, K.P., Andersen, K., Levey, A.I., Backman, V.M., Matthiasdottir, S., Jonsdottir, T., Palsson, S., Einarsdottir, H., Gunnarsdottir, S., Gylfason, A., Vaccarino, V., Hooper, W.C., Reilly, M.P., Granger, C.B., Austin, H., Rader, D.J., Shah, S.H., Quyyumi, A.A., Gulcher, J.R., Thorgeirsson, G., Thorsteinsdottir, U., Kong, A and Stefansson, K. 2007. A common variant on chromosome 9p21 affects the risk of myocardial infarction. *Science.* **316**: 1491–1493.

Hermalin, A.I and Lowry, D. 2011. The age prevalence of smoking among Chinese women: a case of arrested diffusion? Michigan Population Studies Center. Institute for Social Research.

Hodis, H.N., Mack, W.J., Azen, S.P., Alaupovic, P., Pogoda, J.M., La Bree, L., Hemphill, L.C., Kramsch, D.M and Blankenhorn, D.H. 1994. Triglyceride and cholesterol

rich lipoproteins have a differential effect on mild/moderate and severe lesion progression as assessed by quantitative coronary angiography in a controlled trial of lovastatin, *Circulation.* **90**:42-49.

Horne, B.D., Carlquist, J.F., Muhlestein, J.B., Bair, T.L and Anderson, J.L. 2008. Association of Variation in the Chromosome 9p21 Locus With Myocardial Infarction Versus Chronic Coronary Artery Disease. *Circ. Cardiovasc. Genet.* **1**:85–92.

Hooper, L., Summerbell, C, D., Thompson, R. L., Sills, D., Roberts, F. G., Moore, H. J and Smith, G.D. 2012. "Reduced or modified dietary fat for preventing cardiovascular disease". *Cochrane. Database. Syst. Rev*. **5**: CD002137.

Huang, Y., Zhou, J., Ye, H., Xu, L., Le, Y., Yang, X., Xu, W., Huang, X., Lian, J and Duan S. 2013. Relationship between chemokine (C-X-C motif) ligand 12 gene variant (rs1746048) and coronary heart disease: case-control study and meta-analysis. *Gene.* **25**; 521(1):38-44.

Huang, H., Pan, L., Zhang, L and Chen, Y. 2008. Association of single nucleotide polymorphism rs599839 on chromosome 1p13.3 with premature coronary heart disease in a Chinese Han population. *Zhonghua. Yi. Xue. Yi. Chuan. Xue. Za. Zhi* .**25(6)**:686-9.

Humayun, A.A., Shah, S., Alam, S and Hussein, H. 2009. Relationship of body mass index and dyslipidemia in different age groups of male and female populati on of Peshawar. *J. Ayub. Med. Coll. Abbottabad.* **21(2)**.

Hwang, L. C., Tsai, C. H and Chen, T.H. 2006. Overweight and obesity-related metabolic disorders in hospital employees. *J. Formos. Med. Assoc*. **105(1)**: 56-63.

Iqbal, M.P. 2006. Hyperhomocysteinemia and coronary artery disease in Pakistan. *J. Pak. Med. Assoc.* **56**: 282–285.

Iqbal, S.P., Dodani, S and Qureshi, R. 2004. Risk factors and behaviours for coronary artery disease (CAD) among ambulatory Pakistanis. *J. Pak. Med*. Assoc. **54**: 261-266.

Ismail, J., Jafar, T.H., Jafary, F.H., white, F., Faruqui, A.M and Chaturvedi, N.l. 2004. Risk factors for non-fatal myocardial infarction in young South Asian adults. *Heart*. **90**:259-263

Jafar, T.H., Jafary, F.H., Jessani, S and Chaturvedi, N. 2005. Heart disease epidemic in Pakistan: Women and men are at equal risk. *Am. Heart. J.* **150(2)**: 221-6.

Javed, A., Aziz, S and Raza, A. 2006. Prevalence of cardiac disease in females with hypertension and diabetes millitus presenting with chest pain. *Pak. Heart. J*. **39**:9-12.

Jayalakshmi, M.K., Prabhu Raj, N., Shanmukhappa, N.J and Smilee Johncy, S. 2011. Effect of sedentary life style on anthropometric and cardiovascular parameters. *Int. J. Bio. Med. Res*. **2(4):** 846-851.

Jha, R. 2011. Routledge Handbook of South Asian Economics. Taylor and Francis group. PP 84- 88.

Jiang, C. Q., Liu, B., Cheung, B.M., Lam, T.H., Lin, J. M., Li Jin, Y., Yue, X. J., Ong, K. L., Tam, S., Wong, K.S., Tomlinson, B., Lam, K. S and Thomas, G.N. 2010. A single nucleotide polymorphism in APOA5 determines triglyceride levels in Hong Kong and Guangzhou Chinese. *Eur. J. Hum. Genet*. **18(11)**: 1255-60.

Jones, C.A., Perera, A., Chow, M., Ho, I., Nguyen, J and Davachi, S. 2009. Cardiovascular disease risk among the poor and homeless. what we know so far. *Curr. Cardiol. Rev*. **5:**69-77.

Joshi, P., Islam, S., Pais, P., Reddy, S., Dorairaj, P., Kazmi, K., Pandey, M.R., Haque, S and Shanti. 2007. "Risk factors for early myocardial infarction in South Asians compared with individuals in other countries," *J.AM.A.* **297**: 286–294.

Jousilahti, P., Vartiainen, E., Pekkanen, J.,Tuomilehto, J., Sundvall, J and Puska, P. 1998. Serum Cholesterol Distribution and Coronary Heart Disease Risk Observations and Predictions among Middle-aged Population in Eastern Finland. *Circulation.* **97**: 1087-1094.

Kaddai, V., Jager, J., Gonzalez, T., Najem-Lendom, R., Bonnafous, S., Tran, A., Le Marchand-Brustel, Y., Gual, P., Tanti, J.F and Cormont, M. 2009. Involvement of TNF-alpha in abnormal adipocyte and muscle sortilin expression in obese mice and humans. *Diabetologia*. **52(5):** 932-40.

Kandula, N.R., Tirodkar, M.A., Lauderdale, D.S., Khurana, N. R., Makoul, G and Baker, D.W. 2010.Knowledge Gaps and Misconceptions About Coronary Heart Disease Among U.S. South Asians. *Am. J. Prev. Med*. **38(4)**: 439–442.

Kannel, W.B. 2000. Risk stratification in hypertension: new insights from the Framingham Study. *Am. J. Hypertens*. **13**: 3-10.

Karvanen, J., Silander, K., Kee, F., Tiret, L., Salomaa, V., Kuulasmaa, K., Wiklund, P.G,, Virtamo, J., Saarela, O., Perret, C., Perola, M., Peltonen, L., Cambien, F., Erdmann, J., Samani, N.J., Schunkert, H., Evans, A. 2009. MORGAM Project. The impact of newly identified loci on coronary heart disease, stroke and total mortality in the MORGAM prospective cohorts. *Genet. Epidemiol.* **33(3)**: 237-46.

Kathiresan, S., Melander, O., Guiducci, C., Surti, A., Burtt, N.P., Rieder, M.J., Cooper, G.M., Roos, C., Voight, B.F., Havulinna, A.S., Wahlstrand, B., Hedner, T., Corella, D., Tai, E.S., Ordovas, J.M., Berglund, G., Vartiainen, E., Jousilahti, P., Hedblad, B., Taskinen, M.R., Newton-Cheh, C., Salomaa, V., Peltonen, L., Groop, L., Altshuler, D.M and Orho-Melander, M. 2008. Six new loci associated with blood low-density lipoprotein cholesterol, high-density lipoprotein cholesterol or triglycerides in humans. *Nat. Genet*. **40**: 189–197.

Kathiresan, S., Voight, B. F., Purcell, S., Musunuru, K., Ardissino, D., Mannucci, P. M., Anand, S., Engert, J. C., Samani, N. J., Schunkert, H., Erdmann, J., Reilly, M.P., Rader, D. J., Morgan, T., Spertus, J. A., Stoll, M., Girelli, D., Mc Keown, P.P.,

Patterson, C.C., Siscovick D, S., O'Donnell, C. J., Elosua, R., Peltonen, L., Salomaa, V., Schwartz, S. M., Melander, O., Altshuler, D., Ardissino, D., Merlini, P. A., Berzuini, C., Bernardinelli, L., Peyvandi, F., Tubaro, M., Celli, P., Ferrario, M., Fetiveau, R., Marziliano, N., Casari, G., Galli, M., Ribichini, F., Rossi, M., Bernardi, F., Zonzin, P., Piazza, A., Mannucci, P. M., Schwartz, S.M., Siscovick, D. S., Yee, J., Friedlander, Y., Elosua, R., Marrugat, J., Lucas, G., Subirana, I., Sala, J., Ramos, R., Kathiresan, S., Meigs, J. B., Williams, G., Nathan, D. M., Mac Rae, C. A., O'Donnell, C. J., Salomaa, V., Havulinna, A. S., Peltonen, L., Melander, O., Berglund, G., Voight, B. F., Kathiresan, S., Hirschhorn, J. N., Asselta, R., Duga, S., Spreafico, M., Musunuru, K., Daly, M. J., Purcell, S., Voight, B. F., Purcell, S., Nemesh, J., Korn, J. M., Mc-Carroll, S. A., Schwartz, S. M., Yee, J., Kathiresan, S., Lucas, G., Subirana, I., Elosua, R., Surti, A., Guiducci, C., Gianniny, L., Mirel, D., Parkin, M., Burtt, N., Gabriel, S. B., Samani, N. J., Thompson, J. R., Braund, P. S., Wright, B. J., Balmforth, A. J., Ball, S. G., Hall, A. ; Wellcome Trust Case Control Consortium, Schunkert, H., Erdmann, J., Linsel-Nitschke, P., Lieb, W., Ziegler, A., König, I., Hengstenberg, C., Fischer, M., Stark, K., Grosshennig, A., Preuss, M., Wichmann, H. E., Schreiber, S., Schunkert, H., Samani, N. J., Erdmann, J., Ouwehand, W., Hengstenberg, C., Deloukas, P., Scholz, M., Cambien, F., Reilly, M. P., Li, M., Chen, Z., Wilensky, R., Matthai, W., Qasim, A., Hakonarson, H. H., Devaney, J., Burnett, M. S., Pichard, A. D., Kent, K. M., Satler, L., Lindsay, J. M., Waksman, R., Knouff, C. W., Waterworth, D. M., Walker, M. C., Mooser, V., Epstein, S. E., Rader, D. J., Scheffold, T., Berger, K., Stoll, M., Huge, A., Girelli, D., Martinelli, N., Olivieri, O., Corrocher, R., Morgan, T., Spertus, J. A., Mc-Keown, P., Patterson, C. C., Schunkert, H., Erdmann, E., Linsel-Nitschke, P., Lieb, W., Ziegler, A., König, I. R., Hengstenberg, C., Fischer, M., Stark, K., Grosshennig, A., Preuss, M., Wichmann, H. E., Schreiber, S., Hólm, H., Thorleifsson, G., Thorsteinsdottir, U., Stefansson, K., Engert, J. C., Do, R., Xie, C., Anand, S., Kathiresan, S., Ardissino, D., Mannucci, P. M., Siscovick, D., O'Donnell, C. J., Samani, N. J., Melander, O., Elosua, R., Peltonen, L., Salomaa, V., Schwartz, S. M and Altshuler, D. 2009. Genome-wide association of early-onset myocardial

infarction with single nucleotide polymorphisms and copy number variants. *Nat. Genet.* **41**: 334–341.

Katzmarzyk, P.T., Church, T.S., Craig, C.L and Bouchard, C. 2009. Sitting Time and Mortality from All Causes, Cardiovascular Disease, and Cancer. *Med Sci Sports Exerc.* **41(5)**: 998–1005.

Kearney, P. M., Whelton, M., Reynolds, K., Muntner, P., Whelton, P. K and He, J. R. 2005. Global burden of hypertension: analysis of worldwide data. *Lancet.* **365 (9455)**: 217–223.

Ken-Dror, G., Goldbourt, U and Dankner, R. 2010. Different effects of apolipoprotein A5 SNPs and haplotypes on triglyceride concentration in three ethnic origins. *Hum. Genet.* **55 (5)**: 300-7.

Khan, H., Hafiz Ullah, M and Haq, I.U. 2005. A hospital based study on frequency of risk factors of coronary artery disease in Peshawar. *J. P. M. I.* **19(3)**: 270-275.

Khuwaja, A.K., Fatmi, Z., Soomro, W.B and Khuwaja, N.K. 2003. Risk factors for cardiovascular disease in school children - a pilot study. *J. Pak. Med. Assoc*. **53**: 396-400.

Kleber, M.E., Renner, W., Grammer, T.B., Linsel-Nitschke, P., Boehm, B.O., Winkelmann, B.R., Bugert, P., Hoffmann, M.M and Marz, W. 2010. Association of the single nucleotide polymorphism rs599839 in the vicinity of the sortilin 1 gene with LDL and triglyceride metabolism, coronary heart disease and myocardial infarction. The Ludwigshafen Risk and Cardiovascular Health Study. *Atherosclerosis.* **209**: 492–497.

Klerk, M., Verhoef, P., Clarke, R., Blom, H.J., Kok, F.J and Schouten, E.G. 2002. MTHFR 677C→T polymorphism and risk of coronary heart disease: a meta-analysis. *J.A.M.A.* **288**: 2023–2031.

Kluijtmans, L.A., van den Heuvel, L.P., Boers, G.H., Frosst, P., Stevens, E.M., van Oost, B.A., den Heijer, M., Trijbels, F.J., Rozen, R and Blom, H.J. 1996. Molecular

genetic analysis in mild hyperhomocysteinemia: a common mutation in the methylenetetrahydrofolate reductase gene is a genetic risk factor for cardiovascular disease. *Am. J. Hum. Genet*. **58**: 35–41.

Kooner, J. S., Chambers, J. C., Aguilar-Salinas, C. A., Hinds, D. A., Hyde, C. L., Warnes, G. R., Gómez Pérez, F. J., Frazer, K. A., Elliott, P., Scott, J., Milos, P. M., Cox, D. R and Thompson, J.F. 2008. Genome-wide scan identifies variation in MLXIPL associated with plasma triglycerides. *Nat. Genet***. 40***:* 149–151.

Kreatsoulas, C and Anand, S.S. 2010. The impact of social determinants on cardiovascular disease. *Can. J. Cardiol*. **26**: 8-13.

Kulkarni, H.R., Nanda, N.C and Segrest, J.P. 1999. Increased prevalence of smaller and denser LDL particles in Asian Indians. *Arterioscler. Thromb. Vasc. Biol*. **19**: 2749–2755.

Laghari, A.H., Memon, A.N and Memon, M.S. 2010. Malnutrition a risk factor for myocardial infarction in patients with type-2 diabetes. *R.M.J***. 35(1):** 57-60.

Lahoz, C., Schaefer, E.J., Cupples, L.A., Wilson, P.W., Levy, D., Osgood, D., Parpos, S., Pedro-Botet, J., Daly, J.A and Ordovas, J.M. 2001. Apolipoprotein E genotype and cardiovascular disease in the Framingham Heart Study. *Atherosclerosis*. **154**: 529–537.

Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C. Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., Funke, R., Gage, D., Harris, K., Heaford, A., Howland, J., Kann, L., Lehoczky, J., LeVine, R., McEwan, P., McKernan, K., Meldrim, J., Mesirov, J.P., Miranda, C., Morris, W., Naylor, J., Raymond, C., Rosetti, M., Santos, R., Sheridan, A., Sougnez, C., Stange-Thomann, N., Stojanovic, N., Subramanian, A., Wyman, D., Rogers, J., Sulston, J., Ainscough, R., Beck, S., Bentley, D., Burton, J., Clee, C., Carter, N., Coulson, A., Deadman, R., Deloukas, P., Dunham, A., Dunham, I., Durbin, R., French, L., Grafham, D., Gregory, S., Hubbard, T., Humphray, S., Hunt, A., Jones, M., Lloyd, C., McMurray, A., Matthews, L., Mercer, S., Milne, S., Mullikin, J.C., Mungall, A.,

Plumb, R., Ross, M., Shownkeen, R., Sims, S., Waterston, R.H., Wilson, R.K., Hillier, L.W., McPherson, J.D., Marra, M.A., Mardis, E.R., Fulton, L.A., Chinwalla, A.T., Pepin, K.H., Gish, W.R., Chissoe, S.L., Wendl, M.C., Delehaunty, K.D., Miner, T.L., Delehaunty, A., Kramer, J.B., Cook, L.L., Fulton, R.S., Johnson, D.L., Minx, P.J., Clifton, S.W., Hawkins, T., Branscomb, E., Predki, P., Richardson, P., Wenning, S., Slezak, T., Doggett, N., Cheng, J.F., Olsen, A., Lucas, S., Elkin, C., Uberbacher, E., Frazier, M., Gibbs, R.A., Muzny, D.M., Scherer, S.E., Bouck, J.B., Sodergren, E.J., Worley, K.C., Rives, C.M., Gorrell, J.H., Metzker, M.L., Naylor, S.L., Kucherlapati, R.S., Nelson, D.L., Weinstock, G.M., Sakaki Y., Fujiyama, A., Hattori, M., Yada, T., Toyoda, A., Itoh, T., Kawagoe, C., Watanabe, H., Totoki, Y., Taylor, T., Weissenbach, J., Heilig, R., Saurin, W., Artiguenave, F., Brottier, P., Bruls, T., Pelletier, E., Robert, C., Wincker, P., Smith, D.R., Doucette-Stamm, L., Rubenfield, M., Weinstock, K., Lee, H.M., Dubois, J., Rosenthal, A., Platzer, M., Nyakatura, G., Taudien, S., Rump, A., Yang, H., Yu, J., Wang, J., Huang, G., Gu, J., Hood, L., Rowen, L., Madan, A., Qin, S., Davis, R.W., Federspiel, N.A., Abola, A.P., Proctor, M.J., Myers, R.M., Schmutz, J., Dickson, M., Grimwood, J., Cox, D.R., Olson, M.V., Kaul, R., Raymond, C., Shimizu, N., Kawasaki, K., Minoshima, S., Evans, G.A., Athanasiou, M., Schultz, R., Roe, B.A., Chen, F., Pan, H., Ramser, J., Lehrach, H., Reinhardt, R., McCombie, W.R., de la Bastide, M., Dedhia, N., Blöcker, H., Hornischer, K., Nordsiek, G., Agarwala, R., Aravind, L., Bailey, J.A., Bateman, A., Batzoglou, S., Birney, E., Bork, P., Brown, D.G., Burge, C.B., Cerutti, L., Chen, H.C., Church, D., Clamp, M., Copley, R.R., Doerks, T., Eddy, S.R., Eichler, E.E., Furey, T.S., Galagan, J., Gilbert, J.G., Harmon, C., Hayashizaki, Y., Haussler, D., Hermjakob, H., Hokamp, K., Jang, W., Johnson, L.S., Jones, T.A., Kasif, S., Kaspryzk, A., Kennedy, S., Kent, W.J., Kitts, P., Koonin, E.V., Korf, I., Kulp, D., Lancet, D., Lowe, T.M., McLysaght, A., Mikkelsen, T., Moran, J.V., Mulder, N., Pollara, V.J., Ponting, C.P., Schuler, G., Schultz, J., Slater, G., Smit, A.F., Stupka, E., Szustakowski, J., Thierry-Mieg, D., Thierry-Mieg, J., Wagner, L., Wallis, J., Wheeler, R., Williams, A., Wolf, Y.I., Wolfe, K.H., Yang, S.P., Yeh, R.F., Collins, F., Guyer, M.S., Peterson, J.,

Felsenfeld, A., Wetterstrand, K.A., Patrinos, A., Morgan, M.J., de Jong, P., Catanese, J.J., Osoegawa, K., Shizuya, H., Choi, S and Chen, Y.J. 2001. Initial sequencing and analysis of the human genome. *Nature*. **409(6822)**: 860-921.

Lanktree, M., Oh, J and Hegele, R.A. 2008. Genetic testing for atherosclerosis risk: inevitability or pipe dream. *Can. J. Cardiol*. **24 (11)**: 851-4.

Laurila, P.P., Naukkarinen, j., Kristiansson, K., Ripatti, S., Kauttu, T., Silander, K., Salomaa, V., Perola, M., Karhunen, P.J., Barter, P.H., Ehnohol, C and Peltonen, L. 2010. Genetic Association and Interaction Analysis of *USF1* and *APOA5* on Lipid Levels and Atherosclerosis. *Arterioscler. Thromb. Vasc. Biol*. **30**: 346-352.

Lavie, C. J., Milani, R.V and Ventura, H.O. 2009. Obesity and Cardiovascular Disease: Risk Factor, Paradox, and Impact of Weight Loss. *J. Am. Col. Cardiolo***.** **53(21)**:1925–1932.

Levy, D and Thom, T.J. 1998**.** Death rates from coronary disease—progress and a puzzling paradox. *N. Engl. J. Med*. **339**: 915–917.

Lewis, S.J., Ebrahim, S and Smith, G. D. 2005. Meta-analysis of MTHFR 677C->T polymorphism and coronary heart disease: does totality of evidence support causal role for homocysteine and preventive potential of folate. *B.M.J*. **331(7524)**: 1053–1056.

Li, J., Xu, H.W and Zhu, X.Y. 2007. Association of *APOA5* gene polymorphism with levels of lipids and atherosclerotic cerebral infarction, *Chinese. J. Med.Genetic*. **24**: 576–578.

Libby, P. 2001. Current concepts of the pathogenesis of the acute coronary syndromes. *Circulation*. **104(3)**: 365-372.

Linsel-Nitschke, P., Heeren, J., Aherrahrou, Z., Bruse, P., Gieger, C., Illig, T., Prokisch, H., Heim, K., Doering, A., Peters, A., Meitinger, T., Wichmann, H.E., Hinney, A., Reinehr, T., Roth, C., Ortlepp, J.R., Soufi, M., Sattler, A.M., Schaefer, J., Stark, K., Hengstenberg, C., Schaefer, A., Schreiber, S., Kronenberg, F., Samani,

N.J., Schunkert, H and Erdmann, J. 2010. Genetic variation at chromosome 1p13.3 affects sortilin mRNA expression, cellular LDLuptake and serum LDL levels which translates to the risk of coronary artery disease. *Atherosclerosis.* **208**:183–9.

Liu, X., Wang, X., Shen, Y., Wu, L., Ruan, X., Lindpaintner, K., Yusuf, S., Engert, J.C., Anand S Tan, X and Liu, L. 2009. The functional variant rs1048990 in PSMA6 is associated with susceptibility to myocardial infarction in a Chinese population. *Atherosclerosis*. **206(1)**: 199-203.

Liu, Z. K., Hu, M., Baum, L., Thomas, G.N and Tomlinson, B. 2010. Associations of polymorphisms in the apolipoprotein A1/C3/A4/A5 gene cluster with familial combined hyperlipidaemia in Hong Kong Chinese. *Atherosclerosis*. **208(2)**: 427-32.

Livingston, R.J., von Niederhausern, A., Jegga, A., Crawford, D.C., Carlson, C.S., Rieder, M.J., Gowrisankar, S., Aronow, B.J., Weiss, R.B and Nickerson, D.A. 2004. Pattern of sequence variation across 213 environmental response genes. *Genome. Res.* **14(10A)**:1821-31.

Lloyd-Sherlock, P., Ebrahim, S and Grosskurth, H. 2014. Is hypertension the new HIV epidemic. *Int. J. Epidemiol*. **43**: 8.

Lo, W.S., Harano, M., Gawlik, M., Yu, Z., Chen, J., Pun, F.W., Tong, K.L., Zhao, C., Ng, S.K., Tsang, S.Y., Uchimura, N., Stober, G and Xue H. 2007. *GABRB2* Association with Schizophrenia: Commonalities and Differences between Ethnic Groups and Clinical Subtypes. *Biol. Psychiatry.* **61**: 653–660.

Loke, Y.K., Hwang, S.L and Tan, M.H. 1997. Delays in thrombolytic therapy in acute myocardial infarction: an audit from the east coast of peninsular Malaysia. *Ann. Acad. Med. Singapore*. **26(3)**:285–289.

Lowe, J.K., Maller, J.B., Pe'er, I., Neale, B.M., Salit, J., Kenny, E.E., Shea, J.L., Burkhardt, R., Smith, J.G., Ji, W., Noel, M., Foo, J.N., Blundell, M.L., Skilling,

V., Garcia, L., Sullivan, M.L., Lee, H.E., Labek, A., Ferdowsian, H., Auerbach, S.B., Lifton, R.P., Newton-Cheh, C., Breslow, J.L., Stoffel, M., Daly, M. J., Altshuler, D.M and Friedman, J.M. 2009. Genome-wide association studies in an isolated founder population from the Pacific Island of Kosrae. *PLoS. Genet*. **5(2)**: e1000365.

Lye, S-H., Chahil, J.K., Bagali, P., Alex, L., Vadivelu, J., Ahmad, W.A.W., Chan, S.P., Thong, M.K., Zain, S and Mohamed, R. 2013. Genetic Polymorphisms in *LDLR*, *APOB*, *PCSK9* and Other Lipid Related Genes Associated with Familial Hypercholesterolemia in Malaysia. *PLoS. ONE.* **8(4)**: e60729.

Mackay, J and Mensah, G.A. 2004. The Atlas of Heart diseases and stroke. Geneva World health organization and CDC. The Hanway Press. London.

Maitra, A., Dash, D., John, S., Sannappa, P. R., Das, A. P., Shanker, J., Rao, V.R., Sridhara, H and Kakkar, V.V. 2009. A common variant in Chromosome 9p21 associated with coronary artery disease in Asian Indians. *J. Genet.* ***88*****(1)**: 113–118.

Malhotra, S., Gupta, M and Chandra, K.K. 2003. Prehospital delay in patients hospitalized with acute myocardial infarction in the emergency unit of a North Indian tertiary care hospital. *Indian. Heart. J*. **55(4)**:349–353.

Malik, M., Bakir, A., Saab, B. A., Roglic, G and King, H. 2005. Glucose intolerance and associated factors in the multi-ethnic population of the United Arab Emirates: results of a national survey. *Diabetes*. *Res. Clin. Pract*. **69(2)**: 188-95.

Mc Cully, K. S. 2007. Homocysteine, vitamins, and vascular disease prevention. *Am. J. Clin. Nutr*. **86** :1563S–1568S.

Mc Gee, D.L. 2005. The diverse population collaboration. Body mass index and mortality: a meta-analysis based on person-level data from twenty-six observation studies. *Am. Epidemio*l. **15:** 87-97.

Mc Keigue, P.M., Miller, G.J and Marmot, M.G. 1989. Coronary heart disease in SSouth Asians overseas. *J. Clin. Epidemiol.* **42:** 597–609.

Mc Pherson, R., Pertsemlidis, A., Kavaslar, N., Stewart, A., Roberts, R., Cox, D.R., Hinds, D.A., Folsom, A.R., Boerwinkle, E., Hobbs, H.H and Cohen, J.C. 2007. A common allele on chromosome 9 associated with coronary heart disease. *Science*. **316**: 1488-1491.

Mehta, R.H and Eagle, K.A. 1998. Secondary prevention in acute myocardial infarction. *B.M.J*. **316**: 838–842.

Memon, M.A and Samad, A. 1999. Acute myocardial infarction in women. *Pak. J.Card*. **10**: 95–107.

Metz, J. A., Stern, J. S., Kris-Etherton, P., Reusser, M. E., Morris, C. D., Hatton, D. C., Oparil, S., Haynes, R. B., Resnick, L. M., Pi-Sunyer, F. X., Clark, S.,Chester, L., McMahon, M., Snyder, G.W., McCarron and D.A. 2000. A randomized trial of improved weight loss with a prepared meal plan in overweight and obese patients: impact on cardiovascular risk reduction. *Arch. Intern. Med*.**160**:2150–2158.

Miller, M., Stone, N.J., Ballantyne, C., Bittner, V., Criqui, M.H., Insberg, H.N., Goldberg, A.C., Howard, W.J., Jacobson, M.S., Kris-Etherton, P.M., Lennie, T.A., Levi, M., Mazzone, T and Pennathur, S. 2011. Triglycerides and cardiovascular disease: a scientific statement from the American Heart Association. *Circulation*. **123**:2292–333.

Misra, R. 2000. Indian Foods: AAPI's Guide to Nutrition, Health and Diabetes. Sec Ed. Professor & Research Director Center for the Study of Health Disparities (CSHD) Member, Intercollegiate Faculty of Nutrition Texas A&M University. PP16-18.

Mohan, V., Deepa, R., Rani, S. S and Premalatha, G. 2001. Prevalence of coronary artery disease and its relationship to lipids in a selected population in South India: the Chennai Urban Population Study. *J. Am. Coll. Cardiol.* **38:** 683–704.

Mohan, V and pradeepa, R. 2009. Epidemiology of diabetes in different Regions of India. *Health. Administrator*.**1**: 1-18.

Mokdad, A.H., Bowman, B.A., Ford, E.S., Vinicor, F., Marks, J.S and Koplan, J.P. 2002. The continuing epidemicsof obesity and diabetes in the United States. *J. Am. Med. Assoc*. **286**: 1195-1200.

Monteiro, C. A., Moura, E.C., Conde, W.L and Popkin, B.M. 2004. Socioeconomic status and obesity in adult populations of developing countries: a review. *Bull. World. Health. Organ.* **82**:940–946.

Murray, C. J and Lopez, A.D. 1997. Mortality by cause for eight regions of the world: Global Burden of Disease study. *Lancet*. **349**: 1269–1276.

Muendlein, A., Geller-Rhomberg, S., Saely, C.H., Winder, T., Sonderegger, G., Rein, P., Beer, S., Vonbank, A and Drexel, H. 2009. Significant impact of chromosomal locus 1p13.3 on serum LDL cholesterol and on angiographically characterized coronary atherosclerosis. *Atherosclerosis*. **206(2)**:494-9.

Mubotter, T., Kluwe, L., Högel, J., Nguyen, R., Cooper, D.N., Mautner, V.F and Kehrer-Sawatzki, H. 2012. "Non-coding RNA ANRIL and the number of plexiform neurofibromas in patients with NF1 microdeletions". *B.M.C. Mol. Genetic*.**13**: 98.

Nanan, D.J. 2002. The obesity pandemic-implications for Pakistan. *J.P.M.A.* **52**: 342-6.

Nasir, K and Rehan, N. 2001. Epidemiology of cigarette smoking in Pakistan. *Addiction*. **96(12):**1847-1854.

Nishtar, S. 2002. Prevention of coronary heart disease in south Asia. *Lancet*. **360**:1015-8.

Nugent, R. 2008. Chronic diseases in developing countries: Health and economic burdens. *Ann. N. Y. Acad. Sci.* **1136**: 70–79.

Nuri, M.M.H., Nawaz, A., Usman, H. 2006. Aproposed study of risk factors of heart disease in rural population of Punjab (Pakistan) — time to act. *Pak. Heart. J*. **39:** 3–4.

O'Donnell, C.J and Elosua, R. 2008. Cardiovascular Risk Factors. Insights From Framingham Heart Study. *Rev. Esp. Cardiol*. **61**: 299-310.

Okamura, T., Kadowaki, T., Hayakawa, T., Kita, Y., Okayama, A and Ueshima, H .2003. What cause of mortality can we predict by cholesterol screening in the Japanese general population? *J. Intern. Med*. **253**: 169–180.

Ouatou**,** S., Ajjemami, M., Charoute**,** Sefri, H., Ghalim, N., Rhaissi, H., Benrahma, H, Barakat, A and Rouba, H. 2014. Association of APOA5 rs662799 and rs3135506 polymorphisms with arterial hypertension in Moroccan patients. *Lipids. Health. Dis.* **13**:60.

Ozaki, K., Sato, H., Iida, A., Mizuno, H., Nakamura, T., Miyamoto, Y., Takahashi, A., Tsunoda, T., Ikegawa, S., Kamatani, N., Hori, M., Nakamura, Y and Tanaka, T. 2006. A functional SNP in PSMA6 confers risk of myocardial infarction in the Japanese population. . *Nat Genet*. **38(8)**: 921-5.

Palmen, J., Smith, A.J., Dorfmeister, B., Putt, W., Humphries, S.E and Talmud, P.J. 2008. The functional interaction on in vitro gene expression of APOA5 SNPs, defining haplotype APOA52, and their paradoxical association with plasma triglyceride but not plasma apo AV levels. *Biochim. Biophys. Acta.* **1782**: 447–452.

Palmer, A.M., Murphy, N and Graham, A. 2004. Triglyceride-rich lipoproteins inhibit cholesterol efflux to apolipoprotein (apo) A1 from human macrophage foam cells. *Atherosclerosis*. **173:**27-38.

Pappas, G.T., Akhtar, P.J., Gergen, W.C., Hadden and Khan, A.Q. 2001. Health status of the Pakistani population: A health profile and comparison with the United States. *Am. J. Public Health.* **91**: 93-98.

Pasmant, E., Laurendeau, I., Heron, D., Vidaud, M., Vidaud, D and Bieche, I. 2007. Characterization of a germ-line deletion, including the entire INK4/ARF locus, in a melanoma-neural system tumor family: identification of ANRIL, an antisense noncoding RNA whose expression coclusters with ARF: *Cancer. Res*. **67**: 3963–3969.

Peden, J.F, Hopewell, J.C., Saleheen, D., Chambers, J.C., Hager, J., Soranzo, N., Collins, R., Danesh, J., Elliott, P., Farrall, M., Stirrups, K., Zhang, W., Hamsten, A., Parish, S., Lathrop, M., Watkins, H., Clarke, R., Deloukas, P and Kooner ,J.S. 2011. A genome-wide association study in Europeans and South Asians identifies five new loci for coronary artery disease. *Nat. Genet.* **643(4)**:339-44.

Pennacchio, L.A., Olivier, M., Hubacek, J.A., Cohen, J.C., Cox, D.R., Fruchart, J.C., Krauss, R.M and Rubin, E.M. 2001. An apolipoprotein influencing triglycerides in humans and mice revealed by comparative sequencing. *Science*. **294**: 169–173.

Poirier, P and Eckel, R.H. 2002. Obesity and cardiovascular disease. *Curr. Atheroscler. Rep*. **4**: 448–453.

Poirier, P., Giles, T.D., Bray, G.A, Hong, Y., Stern, J.S., Pi-Sunyer, F.X and Eckel, R.H. 2006. "Obesity and cardiovascular disease: pathophysiology, evaluation, and effect of weight loss". *Arterioscler. Thromb. Vasc. Biol.* **26 (5)**: 968–76.

Prasad, D., Kabir, Z., Dash, A.K and Das, B.C. 2011. Abdominal obesity, an independent cardiovascular risk factor in Indian subcontinent: A clinico epidemiological evidence summary. *J. Cardiovasc. Res*. **2 (4)**: 199-205.

Pratipanawatr, T., Rawdaree, P., Chetthakul, T., Bunnag, P., Ngarmukos, C., Benjasuratwong, Y., Leelawatana, R., Kosachunhanun, N., Plengvidhya, N., Deerochanawong, C., Suwanwalaikorn, S., Krittiyawong, S., Mongkolsomlit, S and Komoltri, C. 2013. TDR Working Group. Smoking and death in Thai diabetic patients: the Thailand Diabetic Registry cohort. *J. Med. Assoc. Thai*. **96(3)**: 280-7.

Qi.,L Ma, J., Qi, Q., Hartiala, J., Allayee, H and Campos H. 2011. Genetic Risk Score and Risk of Myocardial Infarction in Hispanics. *Circulation*. **123**: **374-380.**

Qi, L, Qi, Q., Prudente, S., Mendonca, C., Andreozzi, F., di Pietro, N., Sturma, M., Novelli, V., Mannino, G. C, Formoso, G., Gervino, E. V, Hauser, T. H., Muehlschlegel, J. D., Niewczas, M. A., Krolewski, A. S., Biolo, G., Pandolfi, A., Rimm, E., Sesti, G., Trischitta, V., Hu, F and Doria A. 2013. Association between a genetic variant related to glutamic acid metabolism and coronary heart disease in individuals with type 2 diabetes. *J.A.M.A.* **310**: 821-828.

Ramachandran, A., Mary, S., Yamuna, A., Murugesan, N and Snehalatha, C. 2008. High prevalence of diabetes and cardiovascular risk factors associated with urbanization in India. *Diabetes. Care.* **31**: 893–8.

Rankin, J and Bhopal, R. 2001. Understanding of heart disease and diabetes in a South Asian community: cross-sectional study testing the 'snowball' sample method. *Public. Health.* **115** (4)**:** 253-260.

Rapp, J.H., Lespine, A., Hamilton, R.L., Colyvas, N., Chaumeton, A.H., Tweede-Hardmann, J., Kotite, L., Kunitake, S.T., Havel, R.J and Kane, J.P. 1994. Triglyceride-rich lipoproteins isolated by selected-affinity anti-apolipoprotein B immunoabsorption from human atherosclerotic plaque. *Arterioscler. Thromb. Vasc. Biol.***14:**1767-1774.

Raymond, M and Rousset, F. 1995. Genepop (Version-1.2)—population-genetics software for exact tests and ecumenicism. *J. Hered*. **86**:248–249.

Reaven, G.M and Laws, A. 1999. Insulin Resistance: The Metabolic Syndrome X. Humanna Press Inc. PP 68.

Rebecca, C., Thurston, L., Kubzansky, D., Kawachi, I and Berkman, F. 2005. Is association between socioeconomic Position and Coronary Heart Diseases Stronger in women than in Men. *Am. J. Epidemiol*. **162(1)**: 57-65.

Rees, S.D., Islam, M,, Hydrie, M.Z., Chaudhary, B., Bellary, S., Hashmi, S and Jafar, T.H. 2011. An FTO variant is associated with Type 2 diabetes in South Asian populations after accounting for body mass index and waist circumference. *Diabetic. Med*. **28:** 673–680.

Reikvam, A and Hagen, T.P. 2002. Markedly changed age distribution among patients hospitalized for acute myocardial infarction. *Scand. Cardiovasc. J*. **36 (4)**: 221– 4.

Reis, J.P., Loria, C.M., Lewis, C.E., Powell-Wiley, T.M., Wei, G.S., Carr, J.J,. Terry, J.G and Liu, K. 2013. Association between duration of overall and abdominal obesity beginning in young adulthood and coronary artery calcification in middle age. *J.A.M.A*. **310(3)**: 280-8.

Reslan, O.M and Khalil, R.A. 2012. Vascular effects of estrogenic menopausal hormone therapy. *Rev. Recent. Clin. Trials*. **7(1)**: 47-70.

Ripatti, S., Tikkanen, E., Melander, M.O., Havullinna, A.S., Silander, K., Sharma, A., Guiducci, C., Perola, M., Jula, A., Sinisalo, J., Lokki, M. L., Nieminen, M.S., Melander, O., Salomaa, V., Peltonen, L and Kathiresan, S.A. 2010. Multi locus genetic risk scores for coronary heart disease: case-control and prospective cohort analyses. *Lancet*. **376 (9750)**: 1393–1400.

Risch, N.J. 2000. Searching for genetic determinants in the new millennium. *Nature*. **405**: 847-56.

Rodriguez, C., Borgelm, J., Courtm, F., Cathalam, G., Fornem, T and Piette, J. 2010. CTCF is a DNA methylation-sensitive positive regulator of the INK/ARF locus. Biochem. Biophys. Res. Commun. **392**: 129–134.

Rosamond, W.D., Chambless, L.E., Folsom, A.R., Cooper, L.S., Conwill, D.E., Clegg, L., Wang, C.H and Heiss, G.1998. Trends in the incidence of myocardial infarction and in mortality due to coronary heart disease. *N. Engl. J. Med.* **339**: 861.

Rosamond,W., Flegal, K., Furie, K., Go, A., Greenlund, K., Haase, N., Hailpern, S.M., Ho, M., Howard, V., Kissela, B., Kittner, S., Lloyd-Jones, D., McDermott, M., Meigs, J., Moy, C., Nichol, G., O'Donnell, C., Roger, V., Sorlie, P., Steinberger, J., Thom, T., Wilson, M and Hong, Y. 2008. Heart disease and stroke statistics--2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation.* **117**: e25–146

Rosendorff, C., Black, H.R., Cannon, C.P., Gersh, B.J., Gore, J., Izzo, J.L., O'Gara, P.T., Oparil, S. 2007. Treatment of Hypertension in the Prevention and Management of Ischemic Heart Disease. *Hypertension*. **50**: e28-e55.

Roeters van Lennep, J. E., Westerveld, H.T., Erkelens, D.W and van der Wall, E.E. 2002. Risk factors for coronary heart disease: implications of gender. *Cardiovasc. Res*. **53:** 538–549.

Russell, M.W., Huse, D.M., Drowns, S., Hamel, E.C and Hartz, S.C. 1998. Direct medical costs of coronary artery disease in the United States. *Am. J. Cardiol*. **81**:1110–5.

Ryan, T.J., Antman, E.M., Brooks, N.H., Califf, R.M., Hillis, L.D., Hiratzka,L.F.,Rapaport, E., Riegel, B., Russell, R.O., Smith, E.E.,Weaver, W.D., Gibbons, R.J., Alpert, J.S.,Eagle, K.A., Gardner, T.J., Garson, A., Gregoratos, G., Smith, S.C. 1999. Update: ACC/AHA guidelines for the management of patients with acute myocardial infarction. A report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee on Management of Acute Myocardial Infarction). *J. Am. Coll. Cardiol*. **34**:890-911.

Saade, S., Cazier, J. B., Sabbagh, M. G., Youhanna, D. A., Karatani, Y., Hager, J., Yeretzian, J. S., El-Khazen, G., Haber, M., Salloum, A. K., Douaihy, B., Othman, R., Shasha, N., Kabbani, S., Al Bayeh, H., Chammas, E., Farrall, M., Gauguier, D., Platt, D.E and Zalloua, P.A. 2011. Large Scale Association Analysis Identifies

Three Susceptibility Loci for Coronary Artery Disease. *PLoS. One.* **6(12)**: e29427.

Saleheen, D., Soranzo, N., Rasheed, A., Scharnagl, H., Gwilliam, R., Alexander, M., Inouye, M., Zaidi, M., Potter, S., Haycock, P., Bumpstead, S., Kaptoge, S., Di Angelantonio, E., Sarwar, N., Hunt, S.E., Sheikh, N., Shah, N., Samuel, M., Haider, S.R., Murtaza, M., Thompson, A., Gobin, R., Butterworth, A., Ahmad, U., Hakeem, A., Zaman, K.S., Kundi, A., Yaqoob, Z., Cheema, L.A., Qamar, N., Faruqui, A., Mallick, N.H., Azhar, M., Samad, A., Ishaq, M., Rasheed, S.Z., Jooma, R., Niazi, J.H., Gardezi, A.R., Memon, N.A., Ghaffar, A., Rehman, F.U., Hoffmann, M.M., Renner, W., Kleber, M.E., Grammer, T.B., Stephens, J., Attwood, A., Koch, K., Hussain, M., Kumar, K., Saleem, A., Kumar, K., Daood, M.S., Gul, A.A., Abbas, S., Zafar, J., Shahid, F., Bhatti, S.M., Ali, S.S., Muhammad, F., Sagoo, G., Bray, S., McGinnis, R., Dudbridge, F., Winkelmann, B.R., Böehm, B., Thompson, S., Ouwehand, W., März, W., Frossard, P., Danesh, J and Deloukas, P. 2010. Genetic Determinants of Major Blood Lipids in Pakistan Compared with Europeans. *Circ. Cardiovasc*. *Genet*. **3**:348-357.

Samani, N. J., Thompson, J. R., Braund, P. S., Wright, B. J., Balmforth, A. J., Ball, S. G., Hall, A. ; Wellcome Trust Case Control Consortium, Schunkert, H., Erdmann, J., Linsel-Nitschke, P., Lieb, W., Ziegler, A., König, I., Hengstenberg, C., Fischer, M., Stark, K., Grosshennig, A., Preuss, M., Wichmann, H. E., Schreiber, S., Schunkert, H., Samani, N. J., Erdmann, J., Ouwehand, W., Hengstenberg, C., Deloukas, P., Scholz, M., Cambien, F., Reilly, M. P., Li, M., Chen, Z., Wilensky, R., Matthai, W., Qasim, A., Hakonarson, H. H., Devaney, J., Burnett, M. S., Pichard, A. D., Kent, K. M., Satler, L., Lindsay, J. M., Waksman, R., Knouff, C. W., Waterworth, D. M., Walker, M. C., Mooser, V., Epstein, S. E., Rader, D. J., Scheffold, T., Berger, K., Stoll, M., Huge, A., Girelli, D., Martinelli, N., Olivieri, O., Corrocher, R., Morgan, T., Spertus, J. A., Mc-Keown, P., Patterson, C. C., Schunkert, H., Erdmann, E., Linsel-Nitschke, P., Lieb, W., Ziegler, A., König, I. R., Hengstenberg, C., Fischer, M., Stark, K., Grosshennig, A., Preuss, M., Wichmann, H. E., Schreiber, S., Hólm, H., Thorleifsson, G., Thorsteinsdottir, U.,

Stefansson, K., Engert, J. C., Do, R., Xie, C., Anand, S., Kathiresan, S., Ardissino, D., Mannucci, P. M., Siscovick, D., O'Donnell, C. J., Samani, N. J., Melander, O., Elosua, R., Peltonen, L., Salomaa, V., Schwartz, S. M and Altshuler, D. 2009. Genome-wide association of early-onset myocardial infarction with single nucleotide polymorphisms and copy number variants. *Nat. Genet*. **41**: 334–341.

Samani, N.J., Erdmann, J., Hall, A.S., Hengstenberg, C., Mangino, M., Mayer, B., Dixon, R.J., Meitinger, T., Braund, P., Wichmann, H.E., Barrett, J.H., König, I.R., Stevens, S.E., Szymczak, S., Tregouet, D.A., Iles, M.M., Pahlke, F., Pollard, H., Lieb, W., Cambien, F., Fischer, M., Ouwehand, W., Blankenberg, S., Balmforth, A.J., Baessler, A., Ball, S.G., Strom, T.M., Braenne, I., Gieger, C., Deloukas, P., Tobin, M.D., Ziegler, A., Thompson, J.R and Schunkert, H. 2007. Genome wide association analysis of coronary artery disease. *N. Engl. J. Med*. **357**: 443-453.

Sambrook, J., Fritsch, E.R and Maniatis, T. 1989. Molecular cloning, a laboratory manual (3rd). Cold Spring Harbor Laboratory Press Cold Spring Harbor, NY.

Sandhu, M.S., Waterworth, D.M., Debenham, S.L., Wheeler, E., Papadakis, K., Zhao, J.H., Song, K., Yuan, X., Johnson, T., Ashford, S., Inouye, M., Luben, R., Sims, M., Hadley, D., McArdle, W., Barter, P., Kesäniemi, Y.A., Mahley, R.W., McPherson, R., Grundy, S.M., Bingham, S.A., Khaw, K.T., Loos, R.J., Waeber, G., Barroso, I., Strachan, D.P., Deloukas, P., Vollenweider, P., Wareham, N.J and Mooser, V. 2008. LDL-cholesterol concentrations: a genome-wide association study. *Lancet.* **371 (9611)**: 483-91.

Sari, I., Davutoglu, V., Erer, B., Tekbas, E., Ucer, E., Ozer, O., Uyarel, H., Aksoy, M. 2009. Analysis of circadian variation of acute myocardial infarction: afternoon predominance in Turkish population. *Int. J. Clin. Pract.* **63 (1)**:82-6.

Saxena, R., Voight, B.F., Lyssenko, V., Burtt, N.P., de Bakker, P.I., Chen, H., Roix, J.J., Kathiresan, S., Hirschhorn, J.N., Daly, M.J., Hughes, T.E., Groop, L., Altshuler, D., Almgren, P., Florez, J.C., Meyer, J., Ardlie, K., Boström, B.K., Isomaa, B.,

Lettre, G., Lindblad, U., Lyon, H.N., Melander, O., Newton-Cheh, C., Nilsson, P., Orho-Melander, M., Råstam, L., Speliotes, E.K., Taskinen, M.R., Tuomi, T., Guiducci, C., Berglund, A., Carlson, J., Gianniny, L., Hackett, R., Hall, L., Holmkvist, J., Laurila, E., Sjögren, M., Sterner, M., Surti, A., Svensson, M., Svensson, M., Tewhey, R., Blumenstiel, B., Parkin, M., Defelice, M., Barry, R., Brodeur, W., Camarata, J., Chia, N., Fava, M., Gibbons, J., Handsaker, B., Healy, C., Nguyen, K., Gates, C., Sougnez, C., Gage, D., Nizzari, M., Gabriel, S.B., Chirn, G.W., Ma, Q., Parikh, H., Richardson, D., Ricke, D and Purcell S. 2007. Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels. *Science*. **316**:1331–1336.

Schunkert, H., Gotz, A., Braund, P., McGinnis, R., Tregouet, D.A., Mangino, M., Linsel-Nitschke, P., Cambien, F., Hengstenberg, C., Stark, K., Blankenberg, S., Tiret, L., Ducimetiere, P., Keniry, A., Ghori, M.J., Schreiber, S., El Mokhtari, N.E., Hall, A.S., Dixon, R.J., Goodall, A.H., Liptau, H., Pollard, H., Schwarz, D.F., Hothorn, L.A., Wichmann, H.E., König, I.R., Fischer, M., Meisinger, C., Ouwehand, W., Deloukas, P., Thompson, J.R., Erdmann, J., Ziegler, A and Samani, N.J. 2008. Repeated replication and a prospective meta-analysis of the association between chromosome 9p21.3 and coronary artery disease. *Circulation*. **117**:1675–1684.

Schunkert, H., König, I.R., Kathiresan, S., Reilly, M.P., Assimes, T.L., Holm, H., Preuss, M., Stewart, A.F., Barbalic, M., Gieger, C., Absher, D., Aherrahrou, Z., Allayee, H., Altshuler, D., Anand, S.S., Andersen, K., Anderson, J.L., Ardissino, D., Ball, S.G., Balmforth, A.J., Barnes, T.A., Becker, D.M., Becker, L.C., Berger, K., Bis, J.C., Boekholdt, S.M., Boerwinkle, E., Braund, P.S., Brown, M.J., Burnett, M.S., Buysschaert, I., Carlquist, J.F., Chen, L., Cichon, S., Codd, V., Davies, R.W., Dedoussis, G., Dehghan, A., Demissie, S., Devaney, J.M., Diemert, P., Do, R., Doering, A., Eifert, S., Mokhtari, N.E., Ellis, S.G., Elosua, R., Engert, J.C., Epstein, S.E., de Faire, U., Fischer, M., Folsom, A.R., Freyer, J., Gigante, B., Girelli, D., Gretarsdottir, S., Gudnason, V., Gulcher, J.R., Halperin, E., Hammond, N., Hazen, S.L., Hofman, A., Horne, B.D., Illig, T., Iribarren, C., Jones, G.T., Jukema, J.W., Kaiser, M.A., Kaplan, L.M., Kastelein, J.J., Khaw,

K.T., Knowles, J.W., Kolovou, G., Kong, A., Laaksonen, R., Lambrechts, D., Leander, K., Lettre, G., Li, M., Lieb, W., Loley, C., Lotery, A.J., Mannucci, P.M., Maouche, S., Martinelli, .N, McKeown, P.P., Meisinger, C., Meitinger, T., Melander, O., Merlini, P.A., Mooser, V., Morgan, T., Mühleisen, T.W., Muhlestein, J.B., Münzel, T., Musunuru, K., Nahrstaedt, J., Nelson, C.P., Nöthen, M.M., Olivieri, O., Patel, R.S., Patterson, C.C., Peters, A., Peyvandi, F., Qu, L., Quyyumi, A.A., Rader, D.J., Rallidis, L.S., Rice, C., Rosendaal, F.R., Rubin, D., Salomaa, V., Sampietro, M.L., Sandhu, M.S., Schadt, E., Schäfer, A., Schillert, A., Schreiber, S., Schrezenmeir, J., Schwartz, S.M., Siscovick, D.S., Sivananthan, M., Sivapalaratnam, S., Smith, A., Smith, T.B., Snoep, J.D., Soranzo, N., Spertus, J.A., Stark, K., Stirrups, K., Stoll, M., Tang, W.H., Tennstedt, S., Thorgeirsson, G., Thorleifsson, G., Tomaszewski, M., Uitterlinden, A.G., van Rij, A.M., Voight, B.F., Wareham, N.J., Wells, G.A., Wichmann, H.E., Wild, P.S., Willenborg, C., Witteman, J.C., Wright, B.J., Ye, S., Zeller, T., Ziegler, A., Cambien, F., Goodall, A.H., Cupples, L.A., Quertermous, T., März, W., Hengstenberg, C., Blankenberg, S., Ouwehand, W.H., Hall, A.S., Deloukas, P., Thompson, J.R., Stefansson, K., Roberts, R., Thorsteinsdottir, U., O'Donnell, C.J., McPherson, R., Erdmann, J and Samani, N.J. 2011. Large-scale association analysis identifies 13 new susceptibility loci for coronary artery disease. *Nat. Genet.* **43**: 333–338.

Scott, L.J., Mohlke, K.L., Bonnycastle, L.L., Willer, C.J., Li, Y., Duren, W.L., Erdos, M.R., Stringham, H.M., Chines, P.S., Jackson, A.U., Prokunina-Olsson, L., Ding, C.J., Swift, A.J., Narisu, N., Hu, T., Pruim, R., Xiao, R., Li, X.Y., Conneely, K.N., Riebow, N.L., Sprau, A.G., Tong, M., White, P.P., Hetrick, K.N., Barnhart, M.W., Bark, C.W., Goldstein, J.L., Watkins, L., Xiang, F., Saramies, J., Buchanan, T.A., Watanabe, R.M., Valle, T.T., Kinnunen, L., Abecasis, G.R., Pugh, E.W., Doheny, K.F., Bergman, R.N., Tuomilehto, J., Collins, F.S and Boehnke, M. 2007. A genome-wide association study of type 2 diabetes in Finns detects multiple susceptibility variants. *Science*. **316**:1341–1345.

Sehrish, W. 2011. Pakistan at high risk of developing heart diseases. Report. express tribune. Pakistan.

Shahid, A., Kitchlew, A. R and Abbas, S. 2009. Disease Burden of Ischemic Heart Disease in Pakistan and its Risk Factors. *Ann. Pak. Inst. Med. Sci*. **5(3)**: 145-150.

Shen, G.Q., Li, L., Rao, S., Abdullah, K.G., Ban, J.M., Lee, B.S., Park, J.E and Wang, Q.K. 2008. Four SNPs on chromosome 9p21 in a South Korean population implicate a genetic locus that confers high cross-race risk for development of coronary artery disease. *Arterioscler. Thromb. Vasc. Biol*. **28:**360-365.

Shen, G.Q., Rao, S., Martinelli, N., Li, L., Olivieri, O., Corrocher, R., Abdullah, K.G., Hazen, S.L., Smith, J., Barnard, J., Plow, E.F., Girelli, D and Wang, Q.K. 2008. Association between four SNPs on chromosome 9p21 and myocardial infarction is replicated in an Italian population. *J. Hum. Genet*. **53(2)**:144–150.

Siddiuqi, A.H and Kayani, A.Z. 2000. Acute myocardial infarction -Clinical profile of 1000 cases. *Pak. Heart. J*. **4(33)**:1-2 2000.

Sigdel, M., Yadav, B.K., Gyawali P., Regmi, P., Baral, S., Regmi, S.R and Jha, B. 2012. Non-high density lipoprotein cholesterol versus low density lipoprotein cholesterol as a discriminating factor for myocardial infarction. *B.M.C. Resch. Notes*. **5**:640.

Silander, K., Tang, H., Myles, S., Jakkula, E., Timpson, N. J., Cavalli-Sforza, L and Peltonen L. 2009. Worldwide patterns of haplotype diversity at 9p21.3, a locus associated with type 2 diabetes and coronary heart disease. *Genome. Med.* **1(5)**:51.

Steinberger, J and Daniels, S.R. 2003. Obesity, insulin resistance, diabetes, and cardiovascular risk in children: an American Heart Association scientific statement from the Atherosclerosis, Hypertension, and Obesity in the Young Committee (Council on Cardiovascular Disease in the Young) and the Diabetes Committee (Council on Nutrition, Physical Activity, and Metabolism). *Circulation*. **107**:1448-1453.

Stewart, S., Murphy, N., Walker, A., McGuire, A and McMurray, J.J. 2003. The current cost of angina pectoris to the National Health Service in the UK. *Heart.* **89**:848–53.

Suhrcke, M., Nugent, R.A., Stuckler, D and Rozzo, L. 2006. Chonic Disease: an economic perspective. The Oxford Health Alliance; London.

Sultana, S., Hamid, K., Islam, K. M. S and Roy, S. 2010. Assessment of Prescription Pattern of Hypertensive Patient's Prescription. *Euro J. Pub*. **40:** 500-505.

Syed, S., Rahim, M., Javed M and Qureshi, M.A. 2008. Changes in Blood Pressure, BMI and ECG Patterns in Women Using Low-Dose Contraceptives. *J. Coll. Physicians. Surg. Pak.***18 (1)**: 31-36.

Talmud, P.J., Martin, S., Taskinen, M.R., Frick, M.H., Nieminen, M.S., Kesaniemi, Y.A., Pasternack, A., Humphries, S.E and Syvanne, M. 2004. *APOA5* gene variants, lipoprotein particle distribution, and progression of coronary heart disease: results from the LOCAT study. *J. Lipid. Res.* **45**: 750–756.

Tarride, J. E., Lim, M., Meules, M.E., Luo, W., Burke, N., Reilly, D.O., Bowen, J and Goeree, R. 2009. A review of the cost of cardiovascular disease. *Can. J. Cardiol*. **25(6)**: e195–e202.

The International HapMap Consortium. 2007. A second generation human haplotype map of over 3.1 million SNPs. *Nature*. **449**: 851–861.

Thygesen, K., Alpert, J.S., White, H.D., Jaffe, A.S., Apple, F.S., Galvani, M., Katus, H.A., Newby, L.K., Ravkilde, J., Chaitman, B., Clemmensen, P.M., Dellborg, M., Hod, H., Porela, P., Underwood, R., Bax, J.J., Beller, G.A., Bonow, R., Van der Wall, E.E., Bassand, J.P., Wijns, W., Ferguson, T.B., Steg, P.G., Uretsky, B.F., Williams, D.O., Armstrong, P.W., Antman, E.M., Fox, K.A., Hamm, C.W., Ohman, E.M., Simoons, M.L., Poole-Wilson, P.A., Gurfinkel, E.P., Lopez-Sendon, J.L., Pais, P., Mendis, S., Zhu, J.R., Wallentin, L.C., Fernández-Avilés, F., Fox, K.M., Parkhomenko, A.N., Priori, S.G., Tendera, M., Voipio-Pulkki,

L.M., Vahanian, A., Camm, A.J., De Caterina, R., Dean, V., Dickstein, K., Filippatos, G., Funck-Brentano, C., Hellemans, I., Kristensen, S.D., McGregor, K., Sechtem, U., Silber, S., Tendera, M., Widimsky, P., Zamorano, J.L., Morais, J., Brener, S., Harrington, R., Morrow, D., Lim, M., Martinez-Rios, M.A., Steinhubl, S., Levine, G.N., Gibler, W.B., Goff, D., Tubaro, M., Dudek, D and Al-Attar, N. 2007. Universal definition of myocardial infarction. *Circulation*. **116**: 2634-2653.

Thygesen, K., Joseph, S., Alpert and Harvey, D. 2007. Universal definition of myocardial infarction. Joint ESC/ACCF/AHA/WHF Task Force for the Redefinition of Myocardial Infarction. *Eur. Heart. J*. **28**:2525-2538.

Tokuhiro, S., Yamada, R., Chang, X., Suzuki, A., Kochi, Y., Sawada, T., Suzuki, M., Nagasaki, M., Ohtsuki, M., Ono, M., Furukawa, H., Nagashima, M., Yoshino, S., Mabuchi, A., Sekine, A., Saito, S., Takahashi, A., Tsunoda, T., Nakamura, Y and Yamamoto, K. 2003. An intronic SNP in a RUNX1 binding site of SLC22A4, encoding an organic cation transporter, is associated with rheumatoid arthritis. *Nat. Genet.* **35**:341–48.

Topol, E.J., Smith, J., Plow, E.F and Wang, Q.K. 2006. Genetic susceptibility to myocardial infarction and coronary artery disease. *Hum. Mol. Genet.* **15**: R117–R123.

Toyofuku, M., Goto, Y., Matsumoto, T., Miyao, Y., Morii, I., Daikoku, S., Itoh, A., Miyazaki, S and Nonogi, H. 1996. Acute myocardial infarction in young Japanese women. *J. Cardiol*. **28(6)**:313-9.

Tregouet, D.A., König, I.R., Erdmann, J., Munteanu, A., Braund, P.S., Hall, A.S., Grosshennig, A., Linsel-Nitschke, P., Perret, C., DeSuremain, M., Meitinger, T., Wright, B.J., Preuss, M., Balmforth, A.J., Ball, S.G., Meisinger, C., Germain, C., Evans, A., Arveiler, D., Luc, G., Ruidavets, J.B., Morrison, C., van der Harst, P., Schreiber, S., Neureuther, K., Schäfer, A., Bugert, P., El Mokhtari, N.E., Schrezenmeir, J., Stark, K., Rubin, D., Wichmann, H.E., Hengstenberg, C.,

Ouwehand, W., Ziegler, A., Tiret, L., Thompson, J.R., Cambien, F., Schunkert, H and Samani, N.J. 2009. Genome-wide haplotype association study identifies the SLC22A3-LPAL2-LPA gene cluster as a risk locus for coronary artery disease. *Nat. Genet*. **41**: 283–5.

Ueland, P.M and Rozen, R. 2005. *MTHFR* Polymorphisms and Disease. Eorgetown, Austin (TX): Landes Bioscience, Texas U.S.A.

U.S. Department of Health and Human Services. 2004. The health consequences of smoking: a report of the Surgeon General available: http:/www.surgeongeneral.gov/library/smoking consequences.

Van den Hoogen, P.C.W., Feskens, E.J.M., Nagelkerke, N.J., Menotti, A., Nissinen, A and Kromhout, D. 2000. The relation between blood pressure and mortality due to coronary heart disease among men in different parts of the world. Seven Countries Study Research Group. *N. Engl. J. Med*. **342(1)**:1 –8.

Van de Werf, F., Bax, J., Betriu, A., Blomstrom-Lundqvist, C., Crea, F., Falk, V., Filippatos, G., Fox, K., Huber, K., Kastrati, A., Rosengren, A., Steg, P. G, Tubaro, M., Verheugt, F., Weidinger, F and Weis, M. 2008. Management of acute myocardial in patients presenting with persistent ST-segment elevation: the Task Force on the management of ST-segment elevation myocardial infarction of the European Society of Cardiology. *Eur. Heart. J*. **29**: 2909-2945.

van der Net, J.B., Janssens, A.C., Sijbrands, E.J., Steyerberg, E.W. 2009. Value of genetic profiling for the prediction of coronary heart disease. *Am. Heart. J.* **158**:105-110

Vergeer, M., Holleboom, AG., Kastelein, J.J and Kuivenhoven, J.A. 2010. The HDL hypothesis: does high-density lipoprotein protect from atherosclerosis? *J. Lipid. Res.* **51**: 2058–2073.

Voight, B.F., Peloso, G.M., Orho-Melander, M., Frikke-Schmidt, R., Barbalic, M., Jensen, M.K., Hindy, G., Hólm, H., Ding, E.L., Johnson, T., Schunkert, H.,

Samani, N.J., Clarke, R., Hopewell, J.C., Thompson, J.F., Li, M., Thorleifsson, G., Newton-Cheh, C., Musunuru, K., Pirruccello, J.P., Saleheen, D., Chen, L., Stewart, A., Schillert, A., Thorsteinsdottir, U., Thorgeirsson, G., Anand, S., Engert, J.C., Morgan, T., Spertus, J., Stoll, M., Berger, K., Martinelli, N., Girelli, D., McKeown, P.P., Patterson, C.C., Epstein, S.E., Devaney, J., Burnett, M.S., Mooser, V., Ripatti, S., Surakka, I., Nieminen, M.S., Sinisalo, J., Lokki, M.L., Perola, M., Havulinna, A., de Faire, U., Gigante, B., Ingelsson, E., Zeller, T., Wild, P., de Bakker, P.I., Klungel, O.H., Maitland-van der Zee, A.H., Peters, B.J., de Boer, A., Grobbee, D.E., Kamphuisen, P.W., Deneer, V.H., Elbers, C.C., Onland-Moret, N.C., Hofker, M.H., Wijmenga, C., Verschuren, W.M., Boer, J.M., van der Schouw, Y.T., Rasheed, A., Frossard, P., Demissie, S., Willer, C., Do, R., Ordovas, J.M., Abecasis, G.R., Boehnke, M., Mohlke, K.L., Daly, M.J., Guiducci, C., Burtt, N.P., Surti, A., Gonzalez, E., Purcell, S., Gabriel, S., Marrugat, J., Peden, J., Erdmann, J., Diemert, P., Willenborg, C., König, I.R., Fischer, M., Hengstenberg, C., Ziegler, A., Buysschaert, I., Lambrechts, D., Van de Werf, F., Fox, K.A., El Mokhtari, N.E., Rubin, D., Schrezenmeir, J., Schreiber, S., Schäfer, A., Danesh, J., Blankenberg, S., Roberts, R., McPherson, R., Watkins, H., Hall, A.S., Overvad, K., Rimm, E., Boerwinkle, E., Tybjaerg-Hansen, A., Cupples, L.A., Reilly, M.P., Melander, O., Mannucci, P.M., Ardissino, D., Siscovick, D., Elosua, R., Stefansson, K., O'Donnell, C.J., Salomaa, V., Rader, D.J., Peltonen, L., Schwartz, S.M., Altshuler, D and Kathiresan, S. 2012. Plasma HDL cholesterol and risk of myocardial infarction:a mendelian randomisation study. *Lancet*. **380(9841)**: 572-80.

Wallace, C., Newhouse, S.J., Braund, P., Zhang, F., Tobin, M., Falchi, M., Ahmadi, K., Dobson, R.J., Marcano, A.C., Hajat, C., Burton, P., Deloukas, P., Brown, M., Connell, J.M., Dominiczak, A., Lathrop, G.M., Webster, J., Farrall, M., Spector, T., Samani, N.J., Caulfield, M.J and Munroe, P.B. 2008. Genome-wide association study identifies genes for biomarkers of cardiovascular disease: serum urate and dyslipidemia. *Am. J. Hum. Genet*. **82**:139–149.

Wang, A.Z., Li, L., Zhang, B., Shen, G.Q and Wang, Q.K. 2011. Association of SNP rs17465637 on chromosome 1q41 and rs599839 on 1p13.3 with myocardial infarction in an American Caucasian population. *Ann. Hum. Gene*t. **75(4)**:475-82.

Wang, J., Ban, M.R., Zou, G.Y., Cao, H., Lin, T., Kennedy, B.A., Anand, S., Yusuf, S., Huff, M.W., Pollex, R.L and Hegele, R.A. 2008. Polygenic determinants of severe hypertriglyceridemia. *Hum. Mol. Genet*. **17(18)**: 2894-9.

Wang, J., Xu, D., Wu, X., Zhou, C., Wang, H., Guo, Y and Cao, K. 2011. Polymorphisms of matrix metalloproteinases in myocardial infarction: a meta-analysis. *Heart*. **97(19):**1542-6. 42.

Wasay, M and Jabbar, A. 2009. Fight against chronic diseases (High blood pressure, stroke, diabetes and cancer) in Pakistan; cost effective interventions. *J. Pak. Med. Assoc*. **59**: 196-7.

Wasif, S. 2011. Pakistan at high risk of developing heart diseases. Report express tribune.

Wei, Y., Chen, L., Liu, J and Miao, Y. 2013. Independent Association of 9p21 Locus and Subclinical Atherosclerosis. *Intern. Med*. **3**:1.

Wenger, N.K. 1985. Coronary disease in women. *Annu Rev Med* .**36:** 285-294.

Willcox, B.J., He, Q., Chen, R., Yano, K., Masaki, K.H., Grove, J.S., Donlon, T.A., Willcox, D.C and Curb, J.D. 2006. Midlife risk factors and healthy survival in men. *J.A.M.A*. **296:** 2343–50.

Wild, S., Roglic, G., Green, A., Sicree, R and King, H. 2004. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes. Care.* **27:** 1047-53.

Wilkinson, P., Sayer, J., Laji, K., Grundy, C., Marchant, B., Kopelman, P and Timmis, A.D.1996. Comparison of case fatality in South Asian and white patients after acute myocardial infarction: observational study. *B.M.J.* **312**:1330–1333.

Willer, C.J., Sanna, S., Jackson, A.U., Scuteri, A., Bonnycastle, L.L., Clarke, R., Heath, S.C., Timpson, N.J., Najjar, S.S., Stringham, H.M., Strait, J., Duren, W.L., Maschio, A., Busonero, F., Mulas, A., Albai, G., Swift, A.J., Morken, M.A., Narisu, N., Bennett, D., Parish, S., Shen, H., Galan, P., Meneton, P., Hercberg, S., Zelenika, D., Chen, W.M., Li, Y., Scott, L.J., Scheet, P.A., Sundvall, J., Watanabe, R.M., Nagaraja, R., Ebrahim, S., Lawlor, D.A., Ben-Shlomo, Y., Davey-Smith, G., Shuldiner, A.R., Collins, R., Bergman, R.N., Uda, M., Tuomilehto, J., Cao, A., Collins, F.S., Lakatta, E., Lathrop, G.M., Boehnke, M., Schlessinger, D., Mohlke, K.L and Abecasis, G.R. 2008. Newly identified loci that influence lipid concentrations and risk of coronary artery disease. *Nat. Genet.* **40**: 161–169.

Whincup, P.H., Gilg, J.A., Emberson, J.R., Jarvis, M.J., Feyerabend, C., Bryant, A., Walker, M and Cook D.G. 2004. Passive smoking and risk of coronary heart disease and stroke: prospective study with cotinine measurement. *B.M.J*. **329**: 200–205.

Wilson, P.W., Myers, R.H., Larson, M.G., Ordovas, J.M., Wolf, P.A and Schaefer, E.J. 1994. Apolipoprotein E alleles, dyslipidemia, and coronary heart disease. The Framingham Offspring Study. *J.A.M.A.* **272**:1666–1671.

World Health Organization. 2004. History Layout: Steve Ewart, Sue Hobbs and Reda Sadki. Printed in France.

Wu, F., Chen, Y., Parvez, F., Segers, S., Argos, M., Islam, T., Ahmed, A., Rakibuz-Zaman, M., Hasan, R., Sarwar, G and Ahsan, H. 2013. A prospective study of tobacco smoking and mortality in Bangladesh. *PLoS. One*. **8(3)**: e58516.

Wu, Z., Yao, C., Zhao, D., Wu, G., Wang, W., Liu, J and Zeng, Z. 2004. Cardiovascular disease risk factor levels and their relations to CVD rates in China—results of Sino-MONICA project. *Eur. J. Cardiovasc. Prev. Rehabil*. **11(4)**: 275– 83.

Wung, S.F. 2002. Genetic advances in coronary artery disease. *Medsurg Nurs.* **11(6):**296-300.

Yakub, M., Iqbal, M.P and Iqbal, R. 2010. Dietary Patterns Are Associated with Hyperhomocysteinemia in an Urban Pakistani Population. *J. Nutr.* **140(7)**: 1261-1266.

Yamada, Y., Ichihara, S. and Nishida T. 2008. Molecular genetics of myocardial infarction. *Genomic. Med*. **2**:7-22.

Yang, G., Fan, L., Tan, J., Qi G, Zhang, Y., Samet, J.M., Taylor, C.E., Becker, K and Xu, J. 1999. Smoking in China: findings of the 1996 National Prevalence Survey. *J.A.M.A.* **282(13)**:1247– 53.

Ye, S., Willeit, J., Kronenberg, F., Xu, Q and Kiechl, S. 2008. Association of genetic variation on chromosome 9p21 with susceptibility and progression of atherosclerosis: a population-based, prospective study. *J. Am. Coll. Cardiol*. **52**:378–384.

Yousaf, K.M. 2012. Productivity analysis of Pakistan in the last 50 years. Pakistan's Premier Financial.

Yu, Z., Chen, J.,Shi, H., Stoeber, G., Tsang, S.Y and Xue, H. 2006. Analysis of GABRB2 association with schizophrenia in German population with DNA sequencing and one-label extension method for SNP genotyping. *Clinic. Biochem.* **39:** 210–218.

Yuan, J.M., Ross, R.K., Wang, X.L., Gao, Y.T., Henderson, B.E., Yu, M.C. 1996. Morbidity and mortality in relation to cigarette smoking in Shanghai, China. A prospective male cohort study. *J.A.M.A.* **275:** 1646–1650.

Yusuf, S., Hawken, S., Ounpuu, S., Dans, T., Avezum, A., Lanas, F., McQueen, M., Budaj, A., Pais, P., Varigos, J and Lisheng, L. 2004. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. *Lancet.* **364(9438):** 937-52.

Yusuf, S., Reddy, S., Ounpuu, S and Anand, S. 2001. Global burden of cardiovascular diseases: part I: general considerations, the epidemiologic transition, risk factors, and impact of urbanization. *Circulation.* **104:** 2746-2753.

Zagrosek, V., Lundqvist, B.C., Borghi, C., Cifkova, R., Ferreira, R., Foidard, J.M., Gibbs, J.S., Gohlke- Baerworf, C., Gorenek, B., Lung B., Nihoyannopoulos, P., Pieper, P.G., Presbiero, P., Roos-Hesselink,J.W., Schaufelbergerm M,m Seeland, U., Torracca, L. 2011. ESC Guidelines on the management of cardiovascular diseases during pregnancy. The Task Force on the Management of Cardiovascular Diseases during Pregnancy of the European Society of Cardiology (ESC). *Eur. Heart. J.* **32:** 3147–3197.

Zaykin, D.V., Westfall, P.H., Young, S.S., Karnoub, M.A., Wagner, M.J and Ehm, M.G. 2002. Testing association of statistically inferred haplotypes with discrete and continuous traits in samples of unrelated individuals. *Hum. Hered.* **53**:79–91.

Zeggini, E., Weedon, M.N., Lindgren, C.M., Frayling, T.M., Elliott, K.S., Lango H, Timpson, N.J., Perry, J.R., Rayner, N.W., Freathy, R.M., Barrett, J.C., Shields, B., Morris, A.P., Ellard, S., Groves, C.J., Harries, L.W., Marchini, J.L., Owen, K.R., Knight, B., Cardon, L.R., Walker, M., Hitman, G.A., Morris, A.D., Doney, A.S., McCarthy, M.I and Hattersley, A.T. 2007. Replication of genome-wide association signals in UK samples reveals risk loci for type 2 diabetes. *Science*. **316**:1336–1341.

Zeng, Q., Yuan, Y., Wang, S., Sun, J., Zhang, T and Qi, M. 2013. Polymorphisms on Chromosome 9p21 Confer a Risk for Acute Coronary Syndrome in a Chinese Han Population. *Canad. J. Cardiol*. **1:** 5.

Zhang, L.N., Liu, P.P., Zhou, J., Huang, R.S., Yuan, F., Fei, L.J., Huang, Y., Xu, L., Hao, L.M., Qiu, X.J., Le, Y., Yang, X., Xu, W., Huang, X., Ye, M., Lian, J and Duan, S. 2013. Positive correlation between variants of lipid metabolism-related genes and coronary heart disease. *Mol. Med. Rep*. **8(1)**: 260-6.

ANNEXURE I

Patient No.: _____ Date: _____

Information Performa for Cardiac Patients (myocardial infarction)

Personal Information:

Patients name_____ Father's name _____

Age:_____ Gender :M/F

Marital status:_____ Cousin marriage/out of family

Phone number: _____ Residence number_____

Age at the time of diagnosis:_____ Duration of MI:_____

Postal address_____

_____

_____

Weight:_____height:_____ Body mass index:_____

Number of individuals in house:_____ caste:_____

Educational level:

*Illitrate *up to primary*Secondary to intermediate*Graduate and above

Employment Status:

Job(govt/private) *retired *jobless *Businessman

Monthly house hold income in rupees:

Up to 5000 *10,000-20,000 *<30,000

Socioeconomic status:

Low *Middle *High

Family history

Parents *sister/brother *grandparents or any other

Life style:

Physical activity exercise/walk

Have smoked: yes/no

Present smoking status: *daily *ocassionaly *not smoking at present

Smoking (packs/month): _____

Other complication with heart attack:

Diabetes

hypertension

angina pectoris

Coronary artery surgery (angioplasty, stent, or coronary bypass

Hyperlipidemia

coronary artery obstruction

Nutrition

In a typical day, indicate how many servings you eat or drink of the following:

Breads, cereals, rice :_____ Fruits: _____

Vegetables _____ Dairy products (milk, yogurt)_____

Cups of tea_____ Water (8 oz.) _____

Fast food_____ Red Meat:_____

Chicken _____ fish_____

Which of the following do you typically eat and/or use in cooking? Oil/ghee

How many times in week do you eat out? _____Breakfast Lunch Dinner

Are you currently taking any medication: yes/no

Name of regular used

medicines_____

Heart attack

history:_____

_____

_____

ANNEXURE II

DNA Extraction

Frozen blood samples were thawed at room temperature. TE buffer of (500-700 µl) was mixed with 500 µl blood. mixed by inverting tubes ups and down several times and left at room temperature or 10-15 min. Centrifugation was done at 25ºC with 13500 rpm for 5-10 min. Supernatant was discarded leaving the WBCs pellet at the bottom of 1.5 ml eppendorf, broke the pellet by gentle tapping or vortexing, added 1 ml TE buffer, performed mixing and repeated steps 2 and3 until the WBCs pellet became light pink or clear. The broken clear pellet was dissolved into 375 µl of 3M sodium acetate, 25 µl of 10% SDS and 5-10 µl of proteinase K (10 µg/µl) and incubated at 37ºC overnight in a shaking water bath. One volume of chilled chloroform: isoamyl alcohol (24:1) was added into 3 volumes of digested pellet, mixed by gently inverting eppendorfs ups and down until a milky emulsion was formed and centrifuged at 25ºC with 13500 rpm for 5-10 min. Three layers can be visualized after centrifugation. The upper layer contains DNA, the lower layer chloroform: isoamyl alcohol and the middle whitish layer proteins. The DNA layer was carefully transferred into a new labeled eppendorf avoiding contamination from other layers, mixed with equal volume of chilled absolute ethanol by gently inverting eppendorfs ups and down until DNA threads became visible, left at room temperature for 10 min and centrifuged at 25ºC with 13500 rpm for 5-10 min. (**8**) Supernatant was discarded and the DNA pellet was washed with 500-1000 µl chilled 70% ethanol by centrifuging at 25ºC with 13500 rpm for 5-10 min. (**9)** Ethanol was discarded and the DNA pellet was dried by air keeping eppendorfs open inside the laminar air flow at room temperature for 15-30 min. (**10**) The dried DNA pellet was dissolved into 50-100 µl low TE buffer, incubated in a shaking water bath at 70ºC for 30 min, cooled at room temperature and spinned by brief centrifugation. (**11**) DNA was stored in duplicate at -20ºC or -80ºC after assessing its quality and quantity by spectrophotometery or gel electrophoresis. Then we measured the DNA concentration by nanodrop method and visually after electrophoresis in 1% agarose gel and was stored at -20 °C.

ANNEXURE III

1st PCR (Gradient PCR), Reaction mixture

PCR Components	volume of each well		volume of half plate
• Forward primer (FP)			6.25 µl (Each primer)
• Reverse primer (RP)			6.25 µl (Each primer)
• Master Mix 4 µl		50x	200 µl
• H_2O 14.5µl			705 µl
• Taq pol 0.3 µl			15 µl
• DNA 0.6 µl			30 µl
20 µl			1000µl

A 96-well PCR plate was used for optimization process in which at a time eight set of primers were tested (Figure 3.1 & 3.2). For one PCR plate, reaction mixture was prepared in two eppendorf tubes. Then loaded the reaction mixture to 8 eppendorfs (230 µl to each eppendorf tube and add 6.25 µl of each primer forward & reverse). In PCR plates 117 µl of above mixture was added in column 1 & 7 then separate mixture to 2-6 and 8-12 wells (same primer sample in same row), the final volume of each well was 20µl. Used film to seal the plate and short spin for 7 seconds. Gradient PCR program as described above was used (Figure 1).

1st PCR material

Forward primer	0.5 µl		25 µl
Reverse primer	0.5 µl		25 µl
Master mix	4µl	50x	200 µl
H_2O	14. 1µl	_____	705 µl
Taq pol	0.3 µl		15 µl
DNA	0.6 µl		30 µl
	20 µl		**1000µl**

54◦C **56◦C** **58◦C** **60◦C** **62◦C** **64◦C**

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A(1pp)	1	2	3	4	5	6	7	8	9	10	11	12
B(2pp)	13	14	15	16	17	18	19	20	21	22	23	24
C(3pp)	25	26	27	28	29	30	31	32	33	34	35	36
D(4pp)	37	38	39	40	41	42	43	44	45	46	47	48
E(5pp)	49	50	51	52	53	54	55	56	57	58	59	60
F(6pp)	61	62	63	64	65	66	67	68	69	70	71	72
G(7pp)	73	74	75	76	77	78	79	80	81	82	83	84
H(8pp)	85	86	87	88	89	90	91	92	93	94	95	96

Figure 1: Optimization of primers (gradient PCR at six different temperatures)
pp: pair of primers

2nd PCR Material

Forward primer	0.3 µl		15 µl
Reverse primer	0.3 µl		15 µl
Master Mix	4 µl	50x	200 µl
H_2O	14.5µl		725 µl
Taq pol	0.3 µl		15 µl
DNA	0.6 µl		30 µl
	20 µl		**1000µl**

ANNEXURE IV

Agarose Gel Electrophoresis

It was used to determined the of PCR products (presence or absence) and quantify the size (length of the DNA molecule) of the product.

Materials needed:

Agarose

TAE Buffer

6X Sample Loading Buffer

DNA ladder standard

Electrophoresis chamber

Power supply

Gel casting tray and combs

Recipes: TAE Buffer

4.84 g Tris Base

1.14 ml Glacial Acetic Acid

2 ml 0.5M EDTA (pH 8.0)

maked the total volume up to 1L with water

Added Tris base to ~900 ml H_2O. Added acetic acid and EDTA to solution and mixed. Poured mixture into 1 L graduated cylinder and added H_2O to a total volume of 1 L. For convenience a concentrated stock of TAE buffer of 10X was made and was diluted with water to 1X concentration before use.

<u>6X Sample Loading Buffer</u>

1 ml sterilize H_2O

1 ml Glycerol

Enough bromophenol blue to make the buffer deep blue (~ 0.05mg)

Preparing the Agarose gel

Measure 1 g of Agarose powder and add it to a 500 ml flask. Add 100 ml TAE Buffer to the flask. Heated the agarose in a microwave until the solution became clear. The solution was cooled to about 50-55°C and added 5 µl of Ethidium Bromide (Sigma E7637) in 100 ml solution, swirling the flask occasionally to cool evenly. Place the combs in the gel

casting tray. Poured the melted agarose solution into the casting tray and cooled it until it is solid (it should appear milky white). Carefully pull out the combs. Placed the gel in the electrophoresis chamber. Added enough TAE Buffer so that it's about 2-3 mm of buffer over the gel.

Loading the gel

Added 3 µl of Sample Loading Buffer to 2µl of PCR product on a thin film and mix it. The order of the samples loading was recorded. Carefully pipette 5 µl of each sample Loading Buffer mixture into separate wells in the gel. Pipette 2 µl of the DNA ladder (1kb, Fermentas) into at least one well of on the gel.

Running the gel

Placed the lid on the gel box, connecting the electrodes. Connected the electrode wires to the power supply, making sure the positive (red) and negative (black) were correctly connected. Turned on the power supply, about 100 volts. Checked to make sure the current is running through the buffer by looking for bubbles forming on each electrode. Checked that the current running in the correct direction by observing the movement ofthe blue loading dye. The power run until the blue dye approaches the end of the gel. Turn off the power. Disconnected the wires from the power supply. Remove the lid of the electrophoresis chamber. Carefully removed the tray and gel and DNA bands were observed (BioRad, Gel doc system)

ANNEXURE V

The best method was selected for OLE assay. Following data representing OLE test for one SNP i.e. rs10757278. Following values were calculated by using following formula= $\frac{\text{+ve value- (-ve value)}}{\text{-ve value}} \times 100$ Here +ve is that sample for which +ve florescent dye was used

Table: OLE test results for rs10757278 by using OLE one base extension method.

	one base F		one base R	
	A*	G*	U*	C*
AA	243	192	312	185
GG	237	221	164	308
AG	291	225	305	259
water	115	131	87	139
	128	61	225	46
	122	90	77	169
	176	94	218	120
homozygous	2.531646	15.10417	90.2439	66.48649
heterozygous	22.78481	17.1875	85.97561	40

Here F = forward primer; R = reverse primer, AA,GG,AG = three genotypes of template DNA; highlighted value showing best method for OLE assay is one base reverse

ANNEXURE V continue

Table : Results of OLE assay from OLE plate using method one base reverse for rs10757278

OLE readings (i)

dye	1	2	3	4	5	6	7	8	9	10	11	12
dUTP*	304	204	291	306	289	286	291	115	299	166	268	270
dCTP*	140	93	176	139	175	147	137	181	170	190	172	98
dUTP*	180	219	314	164	253	303	286	105	305	151	283	270
dCTP*	193	119	169	99	167	153	113	98	168	177	181	171
dUTP*	290	192	135	298	284	254	284	295	274	293	292	282
dCTP*	174	171	185	99	147	175	162	143	175	161	189	108
dUTP*	287	218	297	242	214	292	294	285	291	280	289	289
dCTP*	187	196	143	178	180	118	151	158	131	174	180	156
dUTP*	259	116	183	286	305	272	293	298	292	289	269	206
dCTP*	179	179	191	148	148	145	154	175	144	167	138	170
dUTP*	291	192	309	308	302	282	297	301	279	229	118	118
dCTP*	107	90	109	158	159	92	110	174	125	172	94	168
dUTP*	251	124	110	215	292	314	251	288	285	283	274	267
dCTP*	179	114	177	107	111	195	108	152	139	134	95	143
dUTP*	313	316	276	282	197	280	281	284	280	280	180	75
dCTP*	120	174	103	173	183	114	166	140	159	129	136	76

Controls

(a)

Calculated values (ii)

dye	1	2	3	4	5	6	7	8	9	10	11	12
dUTP*	3.07	1.73	2.90	3.10	2.87	2.83	2.90	0.54	3.01	1.23	2.58	2.61
dCTP*	0.84	0.22	1.31	0.83	1.30	0.93	0.80	1.38	1.23	1.49	1.26	0.29
dUTP*	1.41	1.93	3.21	1.20	2.39	3.06	2.84	0.41	3.08	1.02	2.78	2.62
dCTP*	1.53	0.57	1.23	0.30	1.20	1.01	0.49	0.28	1.21	1.33	1.37	1.24
dUTP*	2.89	1.57	0.80	2.99	2.80	2.40	2.80	2.96	2.68	2.93	2.91	2.78
dCTP*	1.29	1.24	1.44	0.30	0.93	1.30	1.13	0.88	1.30	1.11	1.48	0.42
dUTP*	2.84	1.93	2.97	2.25	1.87	2.91	2.94	2.81	2.89	2.75	2.87	2.87
dCTP*	1.46	1.57	0.88	1.34	1.36	0.55	0.98	1.08	0.72	1.29	1.36	1.04
dUTP*	2.47	0.56	1.45	2.83	3.08	2.64	2.93	2.99	2.91	2.88	2.60	1.76
dCTP*	1.36	1.35	1.51	0.95	0.94	0.90	1.02	1.30	0.90	1.19	0.81	1.24
dUTP*	2.90	1.57	3.14	3.12	3.05	2.78	2.98	3.04	2.74	2.06	0.58	0.58
dCTP*	0.40	0.18	0.43	1.08	1.09	0.22	0.44	1.29	0.64	1.26	0.24	1.20
dUTP*	2.36	0.65	0.47	1.87	2.92	3.20	2.36	2.85	2.82	2.80	2.67	2.58
dCTP*	1.35	0.50	1.33	0.40	0.47	1.57	0.42	1.00	0.83	0.76	0.25	0.88
dUTP*	3.19	3.23	2.70	2.78	1.64	2.75	2.77	2.81	2.75	2.75	1.41	0.00
dCTP*	0.58	1.28	0.36	1.28	1.41	0.49	1.18	0.84	1.09	0.70	0.79	0.00

(b)

(Continue)
OLE results (iii)

1	2	3	4	5	6	7	8	9	10	11	12
GA	AA	GA	GA	GA	GA	GA	GG	GA	GG	GA	AA
GG	AA	GA	AA	GA	GA	AA	00	GA	GG	GA	GA
GA	GG	GG	AA	GA	GA	GA	GA	GA	GA	GA	AA
GA	GG	GA	GA	GG	AA	GA	GA	GA	GA	GA	GA
GA	GG	GG	GA	GA	GA	GA	GA	GA	GA	GA	GG
AA	AA	AA	GA	GA	AA	AA	GA	AA	GA	00	GG
GA	00	GG	00	AA	GA	AA	GA	GA	GA	AA	GA
AA	GA	AA	GA	GG	AA	GA	GA	GA	GA	GG	control

(C)

Table: (a) Showing FP values from multilable counter of 192 samples for rs10757278, here last two wells for control (use water). Table (4.7 b) representing calculated values. Highlighted blocks represents value for basic genotypes according to baseline (≤ 0.5 is AA; ≥ 1 is TT) and Table (4.7 C) representing related resulted genotypes after reading values.

ANNEXURE VI

Table 1: Pair-wise LD-based associations with MI. D' values of 8 SNPs of 9p21.3 locus

D'	S1	S2	S3	S4	S5	S6	s7	S8
S1	_	**0.36**	**0.494**	**0.52**	**0.517**	**0.54**	**0.058**	**0.5**
S2	_	_	0.03	0.026	0.036	0.045	0.035	0.016
S3	_	_	_	**0.897**	**0.91**	**0.961**	0.006	**0.426**
S4	_	_	_	-	**1**	**0.993**	0.024	**0.471**
S5	_	_	_	-	-	**0.993**	0.018	**0.497**
S6	_	_	_	-	-	-	0.026	**0.53**
S7	_	_	_	_	_	_	_	0.009

Bold values are D' > 0.05

Table 2: Pair-wise haplotype-based associations with MI. r^2 values of 8 associated SNPs

r^2	S1	S2	S3	S4	S5	S6	S7	S8
S1	_	0.094	0.238	0.231	0.262	0.284	**0.001**	0.229
S2	_	_	0.001	0.001	0.001	0.001	0.001	0
S3	_	_	_	**0.705**	**0.824**	**0.877**	**0**	0.169
S4	_	_	_	_	**0.869**	**0.821**	**0**	0.207
S5	_	_	_	_	_	**0.944**	**0**	0.232
S6	_	_	_	_	_	_	**0**	0.249
S7	_	_	_	_	_	_	_	**0**

Bold values are $r^2 > 0.05$

Table 3 : Pairwise haplotype D' and r^2 values for *APOA5* SNPs

D'	S1	S2	S3	S4
S1	_	**0.50**	**0.976**	**0.922**
S2	_	_	**0.526**	**0.563**
S3	_	_	_	**1.000**
S4	_	_	_	_
r^2	S1	S2	S3	S4
S1	_	0.25	**0.743**	**0.694**
S2	_	_	0.213	**0.259**
S3	_	_	_	**0.636**
S4	_	_	_	_

Bold values: D' > 0.5; $r^{2'} > 0.5$

ANNEXURE V11

Preparation of solutions

1L 1M Tris-HCl Weigh 121.1g tris base (2-Amino-2-hydroxymethyl-propane-1,3-diol), dissolve into 700 ml distilled H_2O, adjust pH to 7.4-8.0, raise final volume to 1L, autoclave and "if possible" filter the solution and store at room temperature.

1L 3M sodium acetate Weigh 246.1 × 3g sodium acetate, dissolve into distilled H_2O, adjust pH to 5.2, raise final volume to 1L, autoclave and "if possible" filter the solution and store at room temperature.

1L 10M NaOH Weigh 40 × 10g sodium hydroxide (NaOH), dissolve into 700 ml distilled H_2O, raise final volume to 1L and store at room temperature.

1L 1M HCl Measure 86.2 ml of 11.6M HCl, dilute into 913.8 ml distilled H_2O and store at room temperature. **Precaution** Add acid into distilled H_2O not distilled H_2O into acid.

1L 0.5M EDTA Weigh 372.2/2g ethylene-diamine-tetraacetic acid (EDTA), dissolve into 700 ml distilled H_2O, adjust pH to 8.0 by using NaOH pellets or 10M NaOH solution, raise final volume to 1L, autoclave and "if possible" filter the solution and store at room temperature. **Note** The dissolution of EDTA is pH-sensitive. EDTA will start dissolving on stirrer only when pH is increased towards 8.0 by the pellet-wise or drop-wise addition of NaOH into the starting mixture. Once EDTA solution becomes clear, stop adding NaOH. If excessive NaOH is added during EDTA dissolution, pH may become greater than 8.0, which will then be adjusted to 8.0 by using 1M HCl.

1L TE buffer (10 mM Tris-HCl, 2 mM EDTA, pH 8.0) Dilute 10 ml of 1M Tris-HCl solution and 4 ml of 0.5M EDTA solution into 500 ml distilled H_2O, raise final volume to 1L and store at room temperature.

50 ml 10% SDS Dissolve 5g sodium dodecyl sulphate (SDS) into 45 ml distilled H_2O, heat up to 68°C for dissolution, adjust pH to 7.2 by using 1M HCl and "if possible" filter the solution and store at room temperature. **Note** Don't use fume hood while preparing SDS solution.

10 ml 10 µg/µl proteinase K Dissolve 100 mg proteinase K into 10 ml distilled H_2O, make aliquots of 100 µl and store at -20°C.

500 ml chloroform: isoamyl alcohol (24:1) Mix 480 ml chloroform into 20 ml isoamyl alcohol and store at 4°C.

500 ml 70% ethanol Mix 350 ml absolute ethanol into 150 ml distilled H_2O and store at 4°C.

100 ml low TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) Dilute 1 ml of 1M Tris-HCl solution and 200 µl of 0.5M EDTA solution into 50 ml distilled H_2O; raise final volume to 100 ml and store at room temperature.

10 ml 10 µg/µl ethidium bromide (EtBR) Dissolve 100 mg EtBR into 10 ml distilled H_2O or low TE buffer, make aliquots of 1 ml, cover tubes with aluminum foil and store at room temperature. **Note** EtBR is a strong mutagen and protection from exposure is needed.

50 ml 6× loading dye Dissolve 0.125g bromophenol blue (0.25%), 0.125g xylene cyanol FF (0.25%) and 15 ml of 100% glycerol (30%) or 20g sucrose (40%) or 7.5g Ficoll type 400 (15%) into distilled H_2O for making final volume up to 50 ml and store at 4°C or -20°C.

50 ml 6× restriction stop/loading dye Dissolve 0.05g bromophenol blue (0.1%), 1g Ficoll type 400 (2%) and 1.86g EDTA (100 mM) into distilled H_2O for making final volume up to 50 ml and store at 4°C or -20°C.

1L 10× TBE buffer Dissolve 108g tris-borate, 55g boric acid and 40 ml of 0.5M EDTA solution into 700 ml distilled H_2O, raise final volume to 100 ml and store at room temperature.

1L 1× TBE buffer Dilute 100 ml of 10× TBE buffer into 900 ml distilled H_2O and store at room temperature.

1 ml 25 mM dNTPs Mix 250 µl of each of 100 mM dNTPs (dATP, dGTP, dCTP and dTTP) for making a 25 mM dNTPs solution containing all four dNTPs in equimolar concentration and store at -20°C.

1 ml 2.5 mM dNTPs Dilute 100 µl of 25 mM dNTPs solution into 900 µl distilled H_2O and store at -20°C.

100 ρmoles oligoes or primers Multiply nmoles of lyophilized oligoes or primers with 10. The answer will provide a µl volume of low TE buffer for dissolution of these lyophilized oligoes or primers for making 100 ρmoles or 100 µM solution and store at -20°C. **Example** If a lyophilized oligo or primer contains 40.2 nmoles, dissolve into 420 µl low TE buffer, concentration of the solution will be 0.1 nmoles, 100 ρmoles or 100 µM.

1 ml 10 ρmoles oligoes or primers Dilute 100 µl of 100 ρmoles oligoes or primers' solution into 900 µl distilled H_2O and store at -20°C.

25 mM $MgCl_2$ and PCR buffer PCR buffers and 25 mM, 50 mM or 100 mM $MgCl_2$ are provided alongwith purchased *Taq* DNA polymerase (10 U/µl) and are used as such. Therefore, recipes for solution preparation and dilutions of these PCR reagents are not given.